Soybean Protein Products as Regulators of Liver Low-Density Lipoprotein Receptors. I. Identification of Active β-Conglycinin Subunits

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The ability of the different soy globulins to upregulate low-density lipoprotein (LDL) uptake and metabolism in human hepatoma cells (Hep G2) was investigated, in an attempt to identify peptide components responsible for the upregulation of the LDL receptor. In parallel, the metabolism of soy globulins, added to the culture medium, was investigated by two-dimensional electrophoresis. After addition of soy globulins, there were no marked changes in the cell protein pattern, as evaluated by general protein staining. By immunodetection, intact 7S components were observed both free in the culture medium and bound to plasma membranes. Inside cells, α + α′ subunits in their native forms were not detectable, whereas most of the β chain was found unchanged. Largely unmodified soybean proteins were detected in a lysate of 11S-treated cells. Incubation of Hep G2 cell with purified α + α′ from 7S sharply increased uptake and degradation of ^{125}I-LDL added to the culture medium, whereas the β chains were ineffective; 7S itself was more active than 11S in this assay. The ability to bring about a specific biological effect such as the observed upregulation of LDL receptors correlates in our test system with the kinetics and/or the extent of catabolism of individual components within the cell.

Keywords: *LDL receptor upregulation; soybean 7S and 11S globulins; Hep G2 cells*

INTRODUCTION

The hypocholesterolemic activity of proteins derived from soybean has been clearly demonstrated in animal models and in man (Carroll, 1991). The reduction of cholesterolemia is apparently associated with an activation of the receptor-mediated catabolism of the major cholesterol carriers in plasma, low-density lipoproteins (LDL) (Lovati et al., 1987; Sirtori et al., 1995). The final mechanism of the plasma cholesterol reduction is as yet undefined. At least two hypotheses can be reasonably put forward, i.e., a direct effect either of the protein components (mainly 7S and 11S globulins or fragments thereof) or, in alternative, of the isoflavones in soybean (daidzein and genistein). Both of these hypotheses are supported by experimental and, in part, by clinical findings (Anderson et al., 1995; Erdman, 1995). We recently demonstrated that, in major clinical studies on plasma cholesterol reduction, isoflavone-poor soybean products were used (Sirtori et al., 1997).

Evidence from this laboratory suggests that human hepatoma cells (Hep G2) show an increased expression of LDL receptors, when incubated in the presence of isolated soy globulins (Lovati et al., 1992). The 7S soy globulin, β-conglycinin, is markedly more effective vs the 11S soy glycinin (Lovati et al., 1992) in the LDL receptor upregulation, being also recognized by a specific uptake and degradation system (Lovati et al., 1996). In vivo, however, large peptides are unlikely to go undigested through the gastrointestinal tract, and to identify the putatively active components, the sources of protein most active in LDL receptor upregulation should be identified.

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A detailed investigation was, therefore, carried out in a standard laboratory model of in vitro grown human liver cells (Hep G2) on the activation of the LDL receptor, evaluating in parallel the fate of the purified β-conglycinin subunits in the same system. Data provided by the investigation allowed us to put forward a hypothesis on the identity of the active moiety of β-conglycinin. This hypothesis was later confirmed by examining the contrasting effects on the cell system of a commercial soy isolate and of a soy mutant depleted of a specific subunit, to be detailed in the following paper (Manzoni et al., 1998).

MATERIALS AND METHODS

Reagents. The established human hepatoma cell line (Hep G2) was obtained from American Type Culture Collection (Rockville, MD). Defatted soybean flour was purchased from Cargill BV (Amsterdam, The Netherlands). Eagle's minimum essential medium (MEM), fetal calf serum, trypsin−EDTA (1×), penicillin (10^5 units/L), streptomycin (100 g/L), tricine buffer (1 mmol/L, pH 7.4), and nonessential amino acid solution (100×) were from Gibco (Madison, WI). Disposable Petri dishes were from Corning Glass Works (Corning, NY). ^{125}Iodine, carrier free, in 100 mmol/L NaOH, was from NEN−DuPont (Boston, MA). Sephadex G25 columns (PD10), Immobiline II monomers, Ampholine and Pharmalyte carrier ampholytes (CA) in the pH ranges 3.5−10, 4−6, and 5−7, GelBond PAG foils, Ultrodex powder, the electrofocusing kit for granulated gel, a Multiphor II horizontal electrophoresis chamber, and an EPS 3500 power supply were from Pharmacia Biotech (Uppsala, Sweden). Acrylamide monomers and catalysts, a Protean II vertical electrophoresis chamber, a Trans Blot cell, and a 1000/500 power supply were from Bio-Rad, Hercules, CA. An antiserum toward total soybean proteins was purchased from Sigma (St. Louis, MO); IgGs reactive toward 7S globulin were purified in our laboratory from

S0021-8561(98)00099-5 CCC: $15.00
Published on Web 06/20/1998

immune rabbit sera (Hurn and Chantler, 1980). ECL reagents, Hy-Bond nitrocellulose membrane, and autoradiographic Hyperfilm were from Amersham (Buckinganshire, U.K.). Poly(vinylidenedifluoride) membrane (PVDF), Immobilon P, was from Millipore (Bedford, MA). All other chemicals were of analytical grade from Merck (Darmstadt, Germany).

Cell Culture. The Hep G2 cells were grown in monolayers in 90 mm diameter Petri dishes and maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 in MEM supplemented with 10% fetal calf serum, nonessential amino acid solution (1% v/v), penicillin (10^5 units/L), streptomycin (0.1 g/L), tricine buffer (20 mmol/L, pH 7.4), $NaHCO_3$ (24 mmol/L), and sodium pyruvate (0.11 g/L).

In the experiments carried out to evaluate the LDL receptor modulation, cells were seeded in 35 mm plastic dishes [$(3-5) \times 10^5$ cells] and used just before reaching confluency, usually 6 days after plating. The medium was changed every 2−3 days.

The digestion pattern of soy globulins was monitored in Hep G2 cells, seeded in 90 mm plastic dishes [$(8-15) \times 10^5$ cells] and grown in MEM plus 5% LPDS containing 500 mg/L either 7S or 11S soy globulin. In these experiments the culture medium was replaced every day. After 96 h of growth, the medium was removed and cells were washed twice with 10 mL of PBS and then processed either for electrophoretic analysis or for the release of bound proteins from cell membranes by heparin.

Release by Heparin of Bound Proteins from Plasma Membranes. To define whether whole 7S soy globulin or a subunit thereof would interact with the specific binding site on the plasma membrane, heparin was used essentially as described elsewhere (Lovati et al., 1996). After removal of the growth medium containig 7S, the cell monolayers were washed with cold PBS and then incubated for 1 h in 15 mL of MEM added with 10 g/L heparin. At the end of the incubation, the medium (MEM plus heparin) was removed and cells were harvested. Medium and cells were processed for electrophoretic analysis. To concentrate the proteins in MEM plus heparin, 2 mL aliquots were precipitated with trichloroacetic acid (TCA), washed with acetone, and dried until use.

Lipoproteins and Lipoprotein-Deficient Serum. LDL ($1.019 < d < 1.063$ kg/L) were isolated by sequential preparative ultracentrifugation (Havel et al., 1955) from the plasma of clinically healthy normolipidemic volunteers. Lipoproteins were labeled according to the method of McFarlane as modified by Bilheimer et al. (1972). Unreacted ^{125}I was removed by dialysis for 24 h at 4 °C in 50 mmol/L Tris buffer, pH 7.4, containing 150 mmol/L NaCl and 0.3 mmol/L EDTA, following separation on a Sephadex G-25 column (PD10). ^{125}I-LDL were sterilized by filtration (Millipore filters, 0.45 mm pore size) and stored at 4 °C until use (less than 10 days after preparation). Human lipoprotein-deficient serum (LPDS) was prepared according to Brown et al. (1974).

Two-Dimensional Electrophoresis. Cells were harvested by scraping in distilled water added with an antiproteinase mix, 1 mL/Petri dish; the suspension was homogenized and lyophilized in 125 μL aliquots. The dry material was dissolved in 50 μL of 8 M urea/2% CA/2% β-mercatoethanol. The protein content in the culture medium was evaluated by the Bradford assay (Bradford, 1976); 50 μg was loaded per sample for Coomassie stain and 10 μg for immunodetection. As previously described, 2 mL of heparin wash from a Petri dish was precipitated with TCA, washed with acetone, dried, and dissolved in 50 μL of urea mix as above.

Polypeptide chains were resolved according to a surface charge parameter, isoelectric point (p*I*), by isoelectric focusing on a immobilized pH gradient (IPG) (Bjellqvist et al., 1982; Righetti, 1990). The pH course over the range 4−10 was nonlinear in order to optimize resolution (Gianazza et al., 1985). The slab was reswollen in 8 M urea and 0.5% (w/v) CA in the same pH range as the IPG. The samples were loaded near the anode and run at 15 °C for 15 000 V·h (Gianazza et al., 1987). The IPG strips from the first dimension (1d) were equilibrated with SDS and embedded on top of SDS−PAGE slabs cast with a polyacrylamide gradient (PAA) 7.5−17.5% T. At the end of the second run, the slabs were either stained with Coomassie blue in acid alcoholic solution or with silver nitrate according to Heukeshoven and Dernick (1986) or electroblotted for specific protein detection.

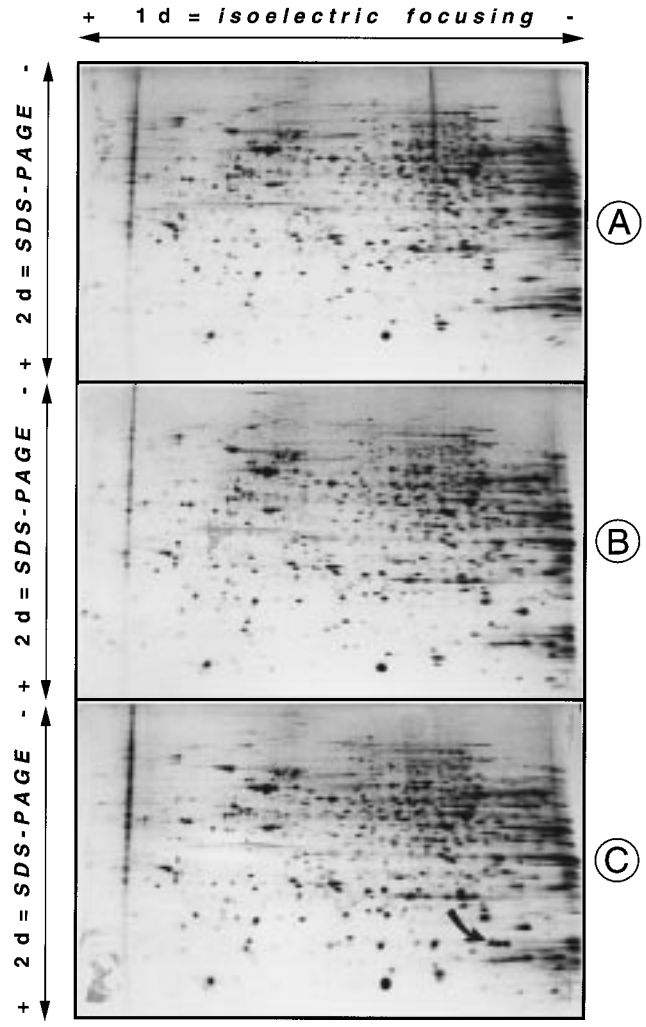

Figure 1. Two-dimensional electrophoresis of total protein extracts from Hep G2 cells grown in different conditions. One-eighth of the cells scraped from a Petri dish at confluence were loaded on an immobilized pH gradient in the range 4−10 [with a nonlinear course according to Gianazza et al. (1985)]. After running for 15 000 V·h and equilibration with SDS, the first-dimension strips were embedded on top of SDS−PAGE slabs with polyacrylamide gradients 7.5−17.5% T. At the end of the run the total protein pattern was revealed by silver staining (Heukeshoven and Dernick 1986). (A) Hep G2 cell grown in the presence of LPDS; (B) in the presence of 7S globulins; (C) in the presence of 11S globulins.

Protein transfer in Towbin buffer (Towbin et al., 1987) was for 4 h at 50 V−400 mA toward PVDF membranes. Lectin-affinity staining was carried out in order to detect mannose-containing glycoproteins, from Hep G2 cells or from 7S globulin. According to Hawkes (1982), the PVDF membranes were incubated with concanavalin A followed by peroxidase [both 50 mg/L in 5% (w/v) BSA in TBS]. Immunostaining was carried out in order to specifically detect soybean globulins or fragments thereof. For this procedure, either anti-7S IgG [purified from immune rabbit serum according to Hurn and Chantler (1980)] at 1:1000 dilution or an anti-total soybean protein antiserum at 1:1000 dilution was followed by peroxidase-conjugated anti-IgG antibodies (raised in goats), also at 1:1000 dilution. For the chromogenic peroxidase zymogram according to Taketa (1987), a solution containig 2 g/L NADH, 0.6 g/L nitroblue tetrazolium, 0.4 g/L phenol, and 3 mL/L H_2O_2

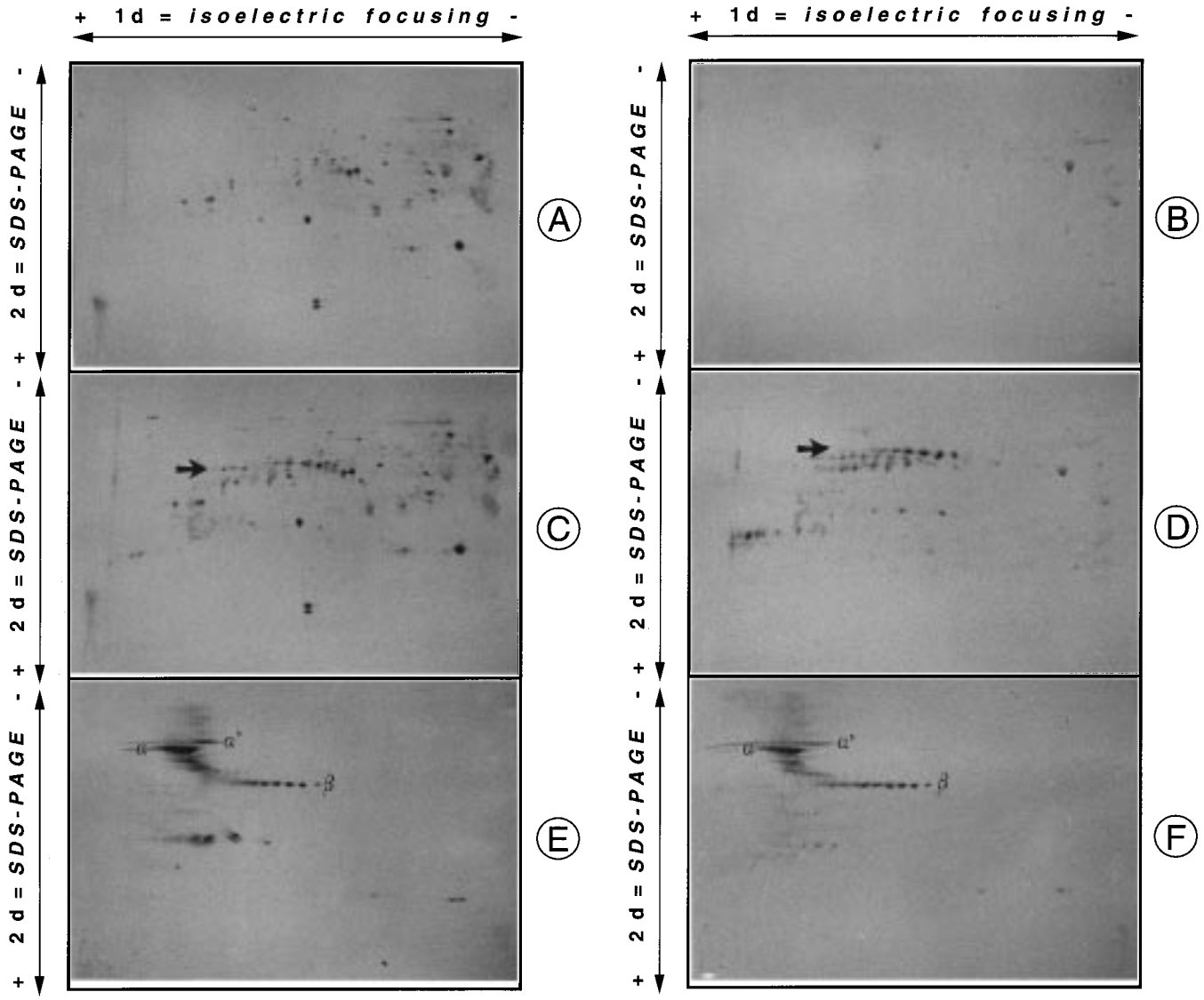

Figure 2. Lectin-affinity blotting and immunoblotting for the two-dimensional maps of extracts from Hep G2 cells grown in different conditions. Proteins from gels as the ones provided in Figure 1 were blotted onto PVDF membranes and probed, in panels A and C, with concanavalin−peroxidase or, in panels B and D, with an anti-7S IgG followed by peroxidase-conjugated anti-immunoglobulin antiserum. Gels A and B correspond to control HepG2, gels C and D to Hep G2 grown in the presence of 7S. In gels E and F, 5 μg of purified 7S was run and blotted in the same conditions and again stained by affinity to concanavalin A (panel E) and immunodetected with anti-7S (panel F).

in phosphate buffer, pH 7.0, was used; for chemiluminescent detection the ECL reagents, with 1 min exposure, were employed.

Extraction of 7S and 11S. The protocol for separation and purification was described in previous reports (Lovati et al., 1992).

Preparative Isoelectric Focusing in a Granulated Bed. This procedure allowed fractionation of $\alpha + \alpha'$ and β subunits from purified 7S globulin. Ultrodex (3.5 g) reswollen in 90 mL of a solution containing 75 mg of 7S globulin purified by ammonium sulfate precipitation (75−100% cut), 32.4 g of urea and 5 mL of CA in the pH range 4−6 were poured in the Pharmacia tray. The slurry was dried with a fan until 22.56 g of water was lost, to bring the final urea concentration to 8 M and the resin near the crack point, as modified from the standard protocols in LKB Application Note 198 in order to account for the presence of urea. After a 20 h run at 8 W and 12 °C, the granulated gel was fractionated with a 30-lane grid. Individual fractions were eluted with 2.5 mL of Tris−glycine buffer pH 8.3, in 8 M urea. The eluates positive for either α or β chains, as checked by SDS−PAGE on a 7.5−17.5% T PAA gradient, were pooled, dialyzed, and liophylized. Prior to use, the powder was dissolved in 8 M urea at a concentration of 10 mg/mL and extensively dialyzed versus pH 7.4 buffer.

Uptake and Degradation of ^{125}I-LDL. Confluent monolayers of cells were preincubated for 24 h at 37 °C in MEM supplemented with 5% LPDS in order to up regulate the LDL receptor (Goldstein and Brown, 1977), in the presence/absence of 7S globulin or of the $\alpha + \alpha'$ and β chains thereof, at 0.05 and 0.5 g/L concentrations. Cell viability, assessed by Trypan blue exclusion, was greater than 95%. A fixed concentration (7.5 mg/L) of ^{125}I-LDL protein was then added to the medium and the incubation was continued for a further 4 h at 37 °C. The nonspecific uptake (binding + internalization) and degradation of ^{125}I-LDL were evaluated in the presence of a 100-fold excess of unlabeled LDL as previously reported (Goldstein et al., 1983). For total uptake (binding + internalization) of ^{125}I-LDL, cell monolayers were directly digested in 1 mol/L NaOH after standard washing procedures. ^{125}I-LDL degradation was measured from the accumulation of noniodide, trichloroacetic acid-soluble ^{125}I in the incubation medium, in excess of that occurring in the absence of cells (Goldstein et al., 1983).

Statistical Analysis. Differences in cell uptake and degradation of LDL after 7S soy globulin incubation were

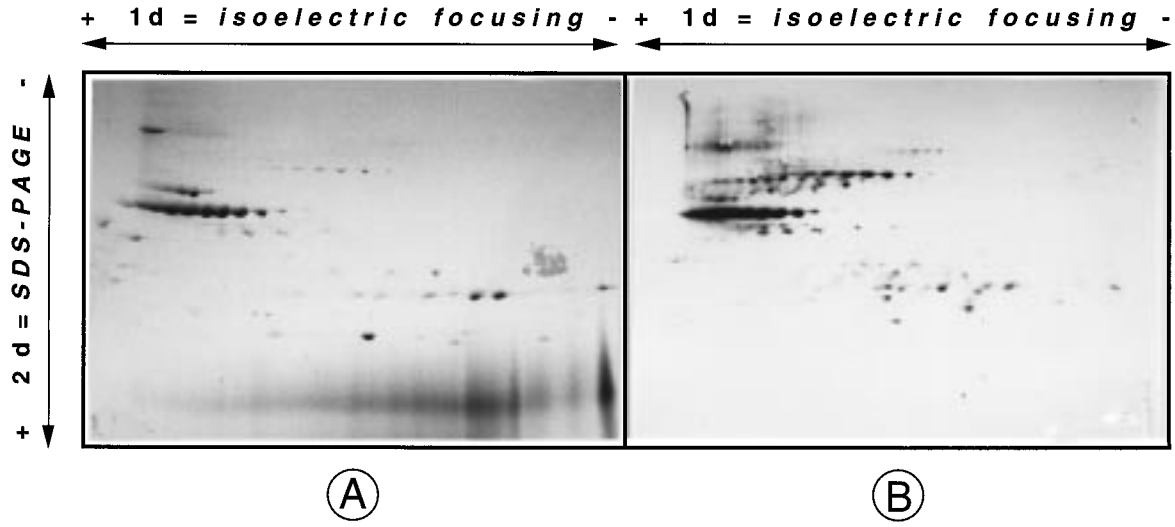

Figure 3. Coomassie stain of control 11S (A) and immunodetection of soybean globulin peptides in cell extracts of Hep G2 cells grown in the presence of 11S (B) upon two-dimensional electrophoresis.

determined with Duncan's multiple range test (Bliss 1967). Differences were considered significant at $P < 0.05$.

RESULTS

Hep G2 cells were exposed to the 7S and 11S isolated soy globulins. By two-dimensional electrophoretic separation (Figure 1) there appeared to be no gross changes in the peptide map of the hepatoma cell lysate. While there was essentially no difference between the peptide patterns of the 7S soy globulin exposed cells (Figure 1B) vs controls (Figure 1A), changes in a few basic peptides of low molecular weight (arrowhead) were observed after cell exposure to 11S soy globulin (Figure 1C), thus suggesting that the cell system had been minimally altered.

When more specific information was searched for, i.e., by evaluating cell *glycoproteins* by a two-dimensional map, before and after cell exposure to soy globulins, striking differences were apparent in the behavior of 7S (Figure 2) and 11S (Figure 3) soy globulin exposed cells. In fact, the glycoprotein pattern of Hep G2 cells (Figure 2A) was markedly altered after exposure to the 7S globulin (Figure 2C), resulting in the presence inside the cells of peptides of a MW around 60 000, with isoelectric points in the neutral to acidic range (arrowhead). Parallel blots probed with an anti-7S antiserum (Figure 2D) clearly indicated that the extra components were in fact slightly modified β-subunits. There were instead no intracellular components identifiable as the major α + α′ subunits of the 7S soy globulin, which appeared to be degraded by the hepatocytes.

As shown in Figure 3, the nonglycoprotein 11S soy globulin was not found to be significantly digested by the Hep G2 cells. Evaluation of immunoreactivity of the protein bands, by comparing isolated 11S with lysates of Hep G2 cells exposed to the globulin, failed to disclose a significant breakdown pattern. Instead, there appeared to be immunoreactive material of a higher molecular weight in cell proteins, possibly related to some minor immunological cross-reactivity.

To evaluate the overall consequence of the interaction of the 7S soy globulin with cell membranes, the peptide pattern of the medium and of cell surface-bound material was also examined, through Coomassie staining and parallel determination of immunoreactivity (Figure 4). The protein material in the medium was made up by albumin and other serum components (Figure 4A), together with some intact 7S globulin (detected by immunoassay in Figure 4B). Most of the protein bound on the cell surfaces and released by heparin was on the contrary recognized by immunoassay as the intact α + α′ and β subunits of 7S globulins (Figure 4D), whereas little serum protein was observed (Figure 4C).

These biochemical findings, also confirming the prior observation of a specific uptake/degradation system for 7S in Hep G2 cells (Lovati et al., 1996), were correlated with functional changes. In particular, the response of Hep G2 cells to the isolated soy globulins and subunits thereof was tested in terms of LDL receptor regulation. Hep G2 cells were exposed to lipoprotein-depleted serum (LPDS) and to different concentrations of the 7S soy globulin and of the separated subunits. LDL receptor activation was evaluated in terms of uptake and degradation of ^{125}I-labeled human LDL. The 7S soy globulin only very slightly affected LDL uptake and degradation in Hep G2 cells at the lower tested concentration, whereas they are increased by about 85% at the higher concentration (respectively 0.05 and 0.5 g/L) (Table 1). While the isolated β subunit does not show any upregulating property, this is not the case with the α + α′, able to increase LDL receptor activity sharply, both when determined as LDL uptake and, somewhat less, as LDL degradation, at 0.5 mg/mL.

DISCUSSION

Nutritional research on the biological consequences of dietary protein intake is particularly active. Studies range from immunological effects of dietary components (Berthou et al., 1987; Chen et al., 1995) to specific biochemical responses, as in this case, leading to potentially useful clinical consequences (Sirtori et al., 1995). Preliminary data from our laboratory had indicated that the isolated 7S soy globulin can actively increase uptake and degradation of the LDL from human serum in liver cell systems (Lovati et al., 1992, 1996), i.e., suggesting a mechanism that might well be responsible for the plasma cholesterol reduction in man. Prior evidence in patients indicates that LDL receptor expression on circulating cells (lymphocytes, where it

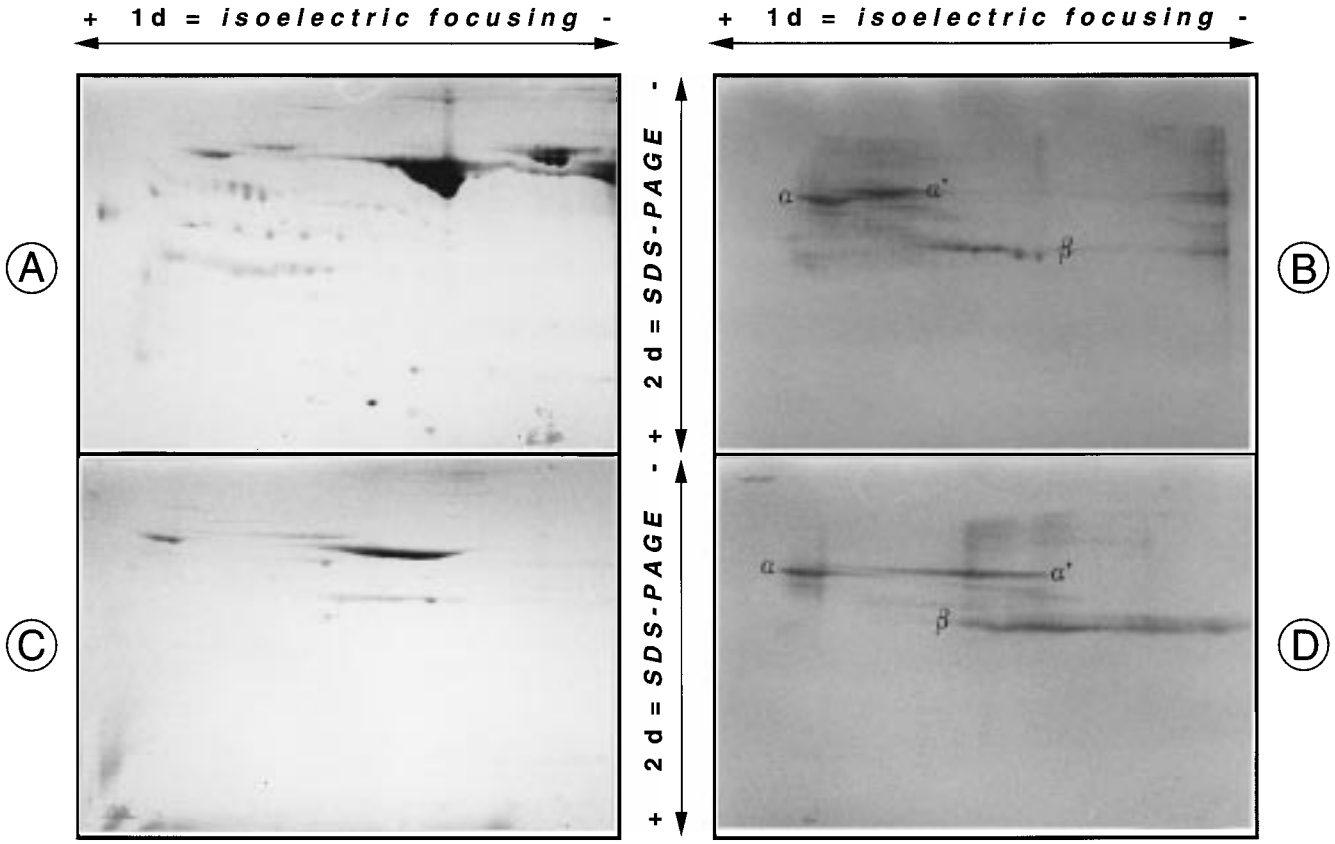

Figure 4. Upper row: Two-dimensional elecrophoresis of the proteins in the incubation medium of cells grown in the presence of 7S globulin. (A) Fifty micrograms was loaded and Coomassie stained; (B) 10 μg was loaded and immunodetected with anti-7S IgG. Lower row: Two-dimensional electrophoresis of the proteins bound to cell membranes and released by heparin treatment. One-seventh of the wash from a Petri dish was concentrated by precipitation with TCA, before running as above. In (C) Coomassie stain; (D) immunoblot.

Table 1. Effect of 7S Soybean Globulin and of Its α + α′ and β Subunits on Uptake (Binding + Internalization) and Degradation of ^{125}I-LDL by a Human Hepatoma Cell Line[a]

		uptake (ng of LDL/mg of cell protein)	degradation (ng of LDL/mg of cell protein)
LPDS		53 ± 6	30 ± 3
7S	0.05 g/L	65 ± 4	34 ± 4
	0.50 g/L	99 ± 8[b]	55 ± 2[b]
α + α′	0.05 g/L	49 ± 5	23 ± 1
	0.50 g/L	225 ± 20[b,c]	90 ± 8[b,c]
β	0.05 g/L	40 ± 4	23 ± 2
	0.50 g/L	38 ± 5	16 ± 3

[a] Confluent monolayers of Hep G2 cells were preincubated for 24 h at 37 °C in MEM with 5% LPDS, in the presence or absence of 7S globulin or α + α′ or β subunits at the listed concentrations. After addition of ^{125}I-LDL (7.5 mg of lipoprotein/L of medium), cells were incubated at 37 °C for further 4 h. Values are means ± SEM of triplicate incubations. Experimental conditions are as described in Materials and Methods. [b] $P < 0.05$ versus LPDS. [c] $P < 0.05$ versus 7S.

parallels that found in hepatocytes) increases after the consumption of soy proteins (Lovati et al., 1987).

In the present studies, the overall protein makeup of Hep G2 cells, a well established human cell line, exposed to isolated 7S soy globulin was first investigated and proved to be essentially unchanged by 2D separation of cellular extracts after 96 h of exposure. Regarding instead the proteolysis of the 7S soy globulin subunits, it could be noted that the α + α′ subunits (the most abundant components of 7S homo- and heterotrimers) in their native forms completely disappeared after cell exposure, whereas most of the β-subunits were unaffected or barely reduced in size. Furthermore, only intact chains were observed in the culture medium; i.e., no proteolysis fragments seemed to be lost from cells and to accumulate in the medium. When the protein material bound at the cell surface was released by heparin treatment and adequately concentrated, immunological identification allowed us to conclude that the whole 7S soy globulin preferentially interacts with heparin-sensitive binding sites, with little competition from the more abundant proteins supplied by the incubation medium (Figure 4C,D).

The potential of separated 7S soy subunits to upregulate LDL receptors in Hep G2 cells was then examined. While the β-subunit (essentially indigested in the test conditions) did not raise LDL receptor expression to any extent, the α + α′ subunits showed powerful LDL receptor stimulating properties.

The selected laboratory method provides an adequate indicator as to the identity of elements responsible for the biological effects. The correlation between the digestion pattern of 7S soy globulin and the activity of the separated components provided a first confirmation to this conclusion and suggested that soluble soy protein preparations, with or without the potentially active components, may be tested in this same system.

The identity of the factor(s) in soy diet responsible for the plasma cholesterol reducing properties is still debated. Many authors maintain that most of the activity is related to the isoflavone content of the commercial formulations (Anderson et al., 1995; Cassidy

et al., 1994; Wang and Murphy, 1994), but in the present system major isoflavones were present to a minimal extent (0.4 g/L daidzein and 0.7 g/L genistein), i.e., similar to products with well-established activity in the clinic (Sirtori et al., 1997). No study has investigated the activity per se of such low concentrations of genistein, a well-known tyrosine kinase inhibitor, on LDL receptor induction. In the presence of LDL receptor activation induced by oncostatin M in Hep G2 cells, high concentrations of the isoflavone (30 g/L) induced a remarkable downregulation (Grove et al., 1991). Other data in similar conditions indicate that the threshold concentration of genistein able to reduce LDL receptor expression is above 2 g/L (Liu et al., 1993). Instead, significant up regulation of the LDL receptors was reported at concentrations between 10 and 20 g/L in liver cells stimulated by hepatocyte growth factor (Kanuck and Ellsoworth, 1995). While it may well be that genistein, similar to estrogens, may have a biphasic effect on LDL receptor expression, on balance it is unlikely that the very low concentrations of isoflavones in unstimulated cells in our system may exert any significant activity on LDL receptors.

The described approach, focusing on the protein components of soy, may well lead, by sort of a "reverse approach", to the identification of the source of small, active peptides. The case may be similar to the one of the recently described hypotensive tripeptides, derived from the digestion of sour milk (Masuda et al., 1996).

ABBREVIATIONS USED

CA, carrier ampholytes; ECL, enhanced chemiluminescence; IPG, immobilized pH gradients for isoelectric focusing; IEF, isoelectric focusing; LDL, low-density lipoproteins; LPDS, lipoprotein-depleted serum; PAA, polyacrylamide; PBS, phosphate-buffered saline; PVDF, poly(vinylidenedifluoride); SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; T, total monomer concentration in a polyacrylamide gel; TBS, Tris-buffered saline; TCA, trichloroacetic acid.

ACKNOWLEDGMENT

We thank Dr. P. A. Murphy, Iowa State University of Food Science and Technology, for the analysis of isoflavone content of the soybean protein preparations.

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Received for review February 4, 1998. Accepted April 30, 1998. This research was supported in part by a grant from the Italian National Research Council (CNR, Rome).

JF980099H