# Soybean Protein Products as Regulators of Liver Low-Density Lipoprotein Receptors. II. α−α′ Rich Commercial Soy Concentrate and α′ Deficient Mutant Differently Affect Low-Density Lipoprotein Receptor Activation

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Activation of low-density lipoprotein (LDL) receptors has been decribed in a human hepatoma cell line (Hep G2) exposed to the 7S globulin from soy (Lovati et al. *J. Agric. Food Chem*. **1998**, *46*, 2474−2480). This in vitro model supports a direct activity of soy protein component(s) in lowering LDL cholesterolemia. To ascertain whether α or α′ subunits from 7S globulin, or both, are responsible for the biochemical effect, a mutant soy cultivar, devoid of the α′ subunit (Keburi), was evaluated in the same model. A commercial isoflavone-poor, heat-hydrolyzed soy preparation found effective in human hypercholesterolemia (Croksoy) was also tested. While Croksoy proved to have a similar activity as the whole 7S globulin, the α′-free Keburi variant showed no effect on LDL receptor activation. These findings support the reliability of the human liver cell model in predicting a human hypocholesterolemic activity and suggest that specific peptide(s) may be responsible. Furthermore, they indicate that some soy cultivars may be best suited for the preparation of products for human nutritional purposes.

**Keywords:** *LDL receptor up regulation; Keburi variant; 7S soybean globulins; Croksoy; Hep G2 cells*

## INTRODUCTION

Reduction of cholesterolemia induced by dietary soy proteins is believed to be associated with an activation of liver LDL receptors in man and experimental animals (Sirtori et al., 1984; Lovati et al., 1987). Recent studies from our group have indicated that *β*-conglycinin (7S globulin) from soy appears to be the component responsible for LDL receptor activation, evaluated in the presence of cultured liver cells (Lovati et al., 1992, 1996).

In view of the growing attention on the mechanism(s) of reduction of cholesterolemia exerted by soy proteins (Sirtori et al., 1998), it appeared of particular interest to examine which soy preparations or soy cultivars provide the protein composition more suitable to induce LDL receptor activation and, as a consequence, a hypocholesterolemic response (Lovati et al., 1987). In the accompanying paper (Lovati et al., 1998) we showed that when a human liver cell line (Hep G2) is presented with 7S globulins, the α + α′ subunits are extensively degraded and, in parallel, LDL receptor activity is markedly stimulated. In contrast, when given to Hep G2 cells, the *β* subunits go largely undigested, as much as they seem unable to activate the LDL receptors.

To examine whether the findings of this first study could be confirmed with other soy protein preparations, the present report explored in detail the activity on LDL receptors of soy proteins from two different sources. We made use of a commercial, isoflavone-free soy protein concentrate (Croksoy) (Sirtori et al., 1997) shown, by clinical experience, to significantly reduce cholesterolemia in man (Sirtori et al., 1998) and compared its activity on LDL receptors of Hep G2 cells with 7S from a variant soy cultivar, the Keburi mutant, deficient in the α′ subunit of *β*-conglycinin (Morita et al., 1994). A number of breeding trials aiming to the manipulation of variant alleles have resulted, in fact, in new soybean varieties with improved processing characteristics (Kitamura, 1995) but whose nutritional/biochemical characteristics are generally unknown.

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## MATERIALS AND METHODS

Most reagents and experimental procedures were as described in the accompanying paper (Lovati et al., 1998).

**Protein Extraction from a Commercial Textured Soy Preparation.** Croksoy (by courtesy of Dr. A. Ferrero, Perfoods, Milano, Italy) is a textured vegetable protein prepared according to a patented procedure (U.S. Patent 4,490,460) making use of rapid heating under high pressure. The total isoflavone content of Croksoy is <0.15 mg/g of dry powder vs ca. 2.5 mg/g in soy flour (Sirtori et al., 1997). Croksoy was processed as previously reported (Lovati et al., 1992). After extraction in 0.03 M Tris, pH 8.0, containing 0.07% v/v *β*-mercaptoethanol, no protein could be precipitated at pH 6.4. More than 90% of the protein was instead precipitated at pH 4.8; indeed, as made clear by the 2D maps of its proteins (in Figure 1B), most material in Croksoy extract is isoelectric at acidic p*I*.

**Protein Extraction from Keburi, a 7S Soybean Variant.** Soybean variety Keburi was obtained from a collection of soybean germplasm; it is characterized by the absence of the α′ subunit of *β*-conglycinin (7S globulin). This mutant line (Morita et al., 1994) was selected at the National Agriculture Research Center, Tsukuba, Ibaraki, Japan, and provided throught the courtesy of Professor Y. Morita (Fuji Oil Co., Ltd., Tsukuba R&D, Osaka, Japan). After removal of the pericarp and grinding, flour was defatted with hexane [soy:solvent 1:20

S0021-8561(98)00100-9 CCC: $15.00 
Published on Web 06/19/1998

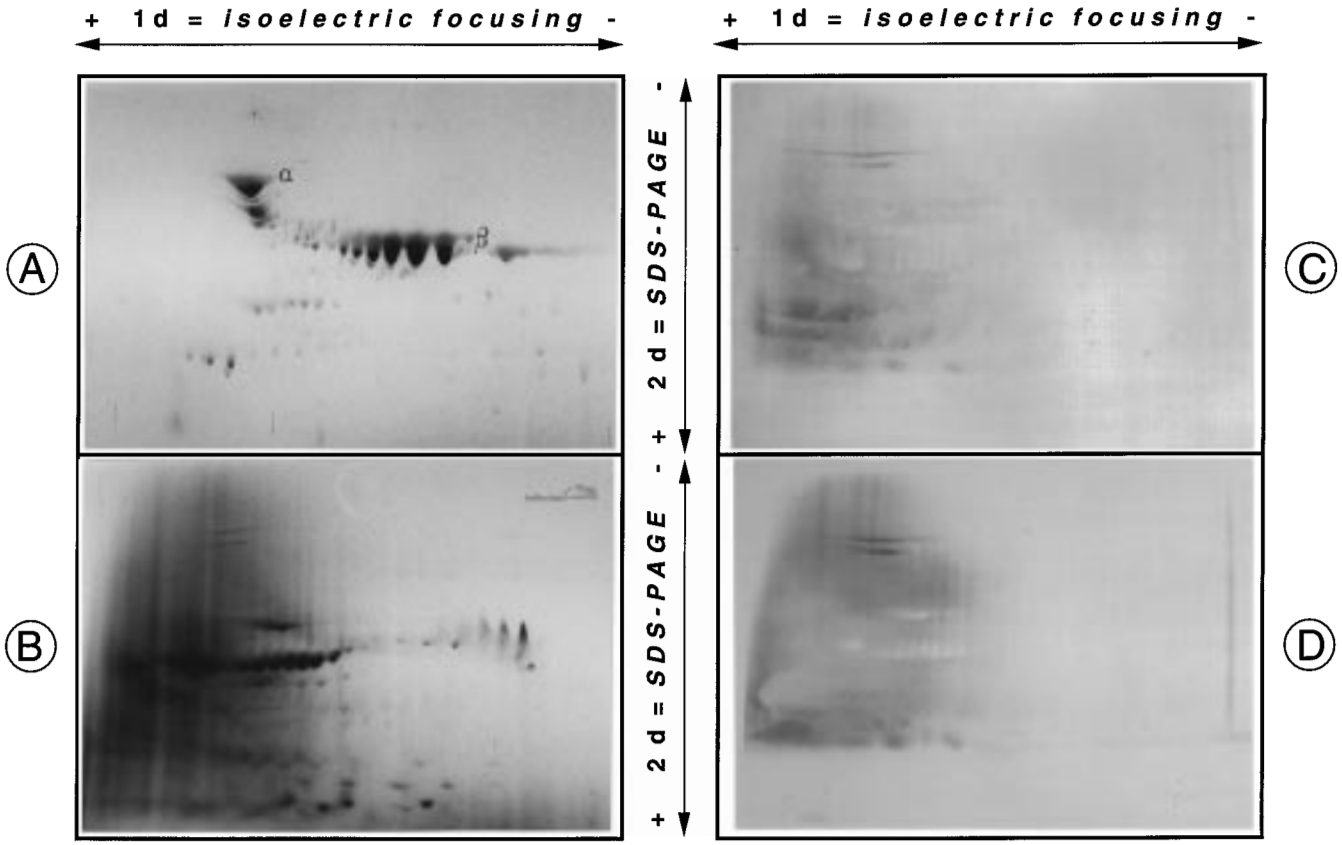


**Figure 1.** Two-dimensional electrophoresis of 7S globulin from the Kebury soybean variety (panel A) and of total protein extracts from Croksoy (panels B−D). Purified 7S (50 $\mu$g) and Croksoy proteins (500 $\mu$g) were loaded on an immobilized pH gradient in the range 4−10. After running for 15 000 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 staining with Coomassie blue in panels A and B. For immunoblotting and lectin-affinity blotting, proteins from Croksoy were blotted onto PVDF membranes and probed, in panel C, with an anti-7S IgG followed by peroxidase-conjugated anti-immunoglobulin antiserum or, in panel D, with concanavalin−peroxidase.

(w/v)] at room temperature until no further lipid was extracted (less than 1%); usually, about three solvent changes were necessary. The separation of 7S globulin from Keburi was performed as described in the Materials and Methods section of the accompanying paper (Lovati et al., 1998).

**Evaluation of the LDL Receptor Activity in Hep G2 Cells.** The protocols for cell culture as well as for the evaluation of uptake and degradation of $^{125}$I-LDL were essentially the same as described in the accompanying paper (Lovati et al., 1998). Confluent monolayers of cells were preincubated for 24 h at 37 °C in MEM supplemented with 5% LPDS in order to upregulate the LDL receptor, in the presence or absence of 7S globulin from reference soybean flour or from Keburi variety or the protein extract from Croksoy, at 0.25, 0.50, and 0.75 g/L concentrations.

**Two-Dimensional Electrophoresis.** The protein content of the two test samples, 7S globulin from variety Keburi and proteins extracted from Croksoy, were characterized by analysis with 2D electrophoresis followed by immuno- and lectin-affinity blotting as detailed in the accompanying paper (Lovati et al., 1998).

**Statistical Analysis.** Difference in cell uptake and degradation of LDL after incubation in the presence of 7S soy globulin, from either a reference flour or from the Keburi mutant, and of Croksoy were determined by ANOVA (SYSTAT, run on a Macintosh LC 630) followed by the Dunnett's test. Differences were considered significant at $P < 0.05$.

## RESULTS

Proteins from Croksoy and the 7S globulin from Keburi soy variety were examined by two-dimensional electrophoresis. In the case of Croksoy, staining for protein and glycoproteins as well as immunostaining for the 7S-related components was carried out. In Figure 1A it clearly appears that 7S from the Keburi variety, as previously indicated by Kitamura (1995), is completely devoid of the α′ subunit. In the case of Croksoy, instead, by immunoblotting against 7S antibodies (Figure 1C) as well as by lectin-affinity blotting (Figure 1D), it is apparent that the major soy globulins have been degraded by the industrial processing. This results in the predominance of peptides in the $M_r$ range around 30 000; a large percentage of the material, as shown by immunodetection and sugar staining, may be identified as 7S-derived peptides.

To confirm or dismiss the hypothesis that the presence of 7S globulin (or its degradation products) and, particularly, of its α subunits is crucial in eliciting LDL receptor activation, the two soy preparations were confronted with cultured Hep G2 cells. Their activity was compared to that of a 7S globulin isolated from standard soybean flour, as used in prior studies (Lovati et al., 1992, 1996). The results, listed in Table 1, confirm the dose-dependent activity of the 7S globulin. Moreover, an almost equivalent activity (on a weight basis) of the Croksoy preparation, rich in 7S globulin degradation products, versus controls, grown in LPDS ($P < 0.05$), is observed. A 73% increase in LDL uptake and degradation is detected following preincubation with the highest concentration of Croksoy (0.75 g/L). In contrast, no LDL receptor activation is observed in the presence of 7S from the Keburi variant: $^{125}$I-LDL

**Table 1. Effect of 7S Globulin from Keburi Soybean Variety and of Croksoy 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 | | 131 ± 6 | 154 ± 3 |
| control 7S | 0.25 g/L | 174 ± 4[b] | 192 ± 4[b] |
| | 0.50 g/L | 202 ± 3[b] | 230 ± 3[b] |
| | 0.75 g/L | 218 ± 1[b] | 250 ± 6[b] |
| Croksoy | 0.25 g/L | 186 ± 4[b] | 213 ± 4[b] |
| | 0.50 g/L | 194 ± 4[b] | 227 ± 3[b] |
| | 0.75 g/L | 206 ± 7[b] | 266 ± 6[b] |
| Keburi 7S | 0.25 g/L | 129 ± 4 | 132 ± 5 |
| | 0.50 g/L | 132 ± 8 | 140 ± 8 |
| | 0.75 g/L | 130 ± 6 | 151 ± 9 |

[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 from soybean variety Keburi or Croksoy 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. [b] $P < 0.05$ versus LPDS.

uptake and degradation were found to be similar to the control values (Table 1).

## DISCUSSION

The results obtained in the present experiments confirm the LDL- receptor upregulation in Hep G2 cells, induced by 7S globulin, and show that this activation is maintained when the cell system is exposed to Croksoy, whose protein pattern is characterized by heat-degraded components (Figure 1B). The Keburi mutant, with null α′ subunit, is unable to modify, even at high concentrations, the LDL receptor pathway in this cell line. These data clearly indicate that the soy protein formulation as well as the β-conglycinin composition differently affect the LDL receptor activity.

Croksoy is routinely used by our group in the dietary treatment of hypercholesterolemic type IIa patients, both adults and children (Sirtori et al., 1998). This dietary replacement has been shown to modify serum and LDL cholesterol concentrations according to baseline cholesterolemia, from a minimum of −3.3% in subjects with cholesterol in the borderline range, way up to −19.6% (LDL cholesterol −24%) in clear-cut hypercholesterolemics (Anderson et al., 1995; Sirtori et al., 1995). The present study was in fact aimed at examining the protein components present in this commercial preparation, as well as their modulating influence on the LDL receptor activity in a cell system already validated by our previous investigations. Protein fragmentation in Croksoy, brought about by heat under pressure, gives rise to a number of peptides, with $M_r$ mainly in the 30 000 range, i.e. similar to the breakdown pattern occurring physiologically during the degradation of soy storage proteins upon seed germination (Kawai et al., 1997).

Keburi, a mutant soybean variety lacking the α′ subunit of β-conglycinin, offered an unique possibility to test which of the α + α′ components in 7S globulin is responsible for the upregulation of the LDL receptor activity, as shown in the accompanying paper (Lovati et al., 1998). The experimental procedure generally utilized for the separation of α from α′ subunit in fact lacks specificity, the two peptides, from a chemical point of view, too similar to permit adequate separation and recovery. It was, therefore, of utmost interest to note the very clear lack of activity of this mutant storage protein on LDL receptor activation. The upregulation was not observed either at the concentrations generally used in our system or at higher concentrations (data not shown).

These data suggest that the LDL receptor activating-cholesterol-lowering properties of soy protein may reside in some specific amino acid stretches of 20−30 amino acids, i.e., either those present in the consensus sequence drawn among all 7S globulins characterized thus far, but not in the β-subunit, or in the 37 amino acid stretch occurring at the $NH_2$ terminal of α′ and not of the α subunit (Wright, 1985, 1988). Such peptides are being synthesized by solid-phase methodology and will be tested in the above-described experimental system. Based on the results of the ongoing experimental studies and on information about structure−function relationships, the design of sequence variants may be also foreseen. These, if found active on LDL receptor modulation, could eventually be introduced into the coding sequence for the α/α′ subunit in the soybean genome by site-directed mutagenesis.

An alternative possibility, suggested by Kurowska and Carroll (1994), is that high proportions of essential amino acids, e.g., lysine and methionine, in the rabbits' diet, are responsible for the induction of hypercholesterolemia by animal protein. This is consequent to an increased hepatic apoprotein B secretion and may be prevented by feeding a high proportion of arginine (Kurowska and Carroll, 1992). The same authors showed that exposure of Hep G2 cells to some of the amino acid mixtures similar to those tested in rabbits induce similar changes in medium apo B (Kurowska and Carroll, 1996). These findings validate the model of Hep G2 cells for studying LDL receptor regulation induced by dietary protein/amino acids but leave open the issue of transferabilty of the in vitro findings to the in vivo protein digestion and intestinal absorption. More detailed knowledge on the sequence/structure of the peptide(s) responsible for the LDL receptor regulation will improve the understanding of this crucial problem. Whatever the case, analysis of the comparative activity of mutant soy proteins, such as the Keburi variant, may lead to a targeted choice of cultivars best suited for the production of soy preparations to be used in the dietary therapy of hypercholesterolemia. It may also possibly help in the understanding of some apparent inconsistencies among the results of different clinical studies on soy protein in hypercholesterolemia (Anderson et al., 1995).

## ABBREVIATIONS USED

IPG, immobilized pH gradients for isoelectric focusing; LDL, low-density lipoproteins; LPDS, lipoprotein deficient serum; MEM, minimum essential medium; Hep G2, human hepatoma cell line

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Received for review February 4, 1998. Accepted April 30, 1998.

JF980100C