Biochemical and Molecular Actions of Nutrients

A Proteomic Investigation of Isolated Soy Proteins with Variable Effects in Experimental and Clinical Studies[1]

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ABSTRACT Dietary preparations of soy proteins used for clinical studies, particularly of hypercholesterolemia, in Europe and the United States were the subject of a proteomic comparison because differences in their composition may explain variability in experimental and clinical results. After two-dimensional electrophoresis, identities of the protein components (globulin subunits and their breakdown products) were established by matrix-assisted laser desorption/ionization mass spectrometry. The soy concentrates (Cholsoy/Croksoy), which were used in most of the Italian and Swiss studies in which reductions in cholesterolemia occurred, exhibited a predominance of breakdown products of the 7S globulin and mainly intact 11S globulin subunits. Soy isolates used in the United States (SUPRO) showed none of the major components corresponding to 7S globulin subunits; only some of the light chains of 11S were intact, and heavy chains of 11S also were fragmented. Ethanol- and nonethanol-treated SUPRO products showed considerable variability in their isoflavone concentrations and there seemed to be differential protein recoveries due to ethanol processing. These findings indicate differences in the protein composition of soy products used in clinical studies. We suggest that standardization should be improved before products are assessed for clinical outcome studies. J. Nutr. 133: 9–14, 2003.

KEY WORDS: • *soy proteins* • *proteomics* • *ethanol extraction* • *hypercholesterolemia* • *soy globulins.*

The increased interest in the clinical use of soy proteins, particularly after the U.S. Food and Drug Administration's approval of their indication in coronary prevention (1), has made it necessary to investigate in more detail the chemical characteristics of preparations used for human nutrition. This is especially important because of the general consensus that protein components are primarily responsible for reducing cholesterolemia (2,3). Hypotheses concerning the role of other components in cholesterolemia, e.g., fibers (4), isoflavones (5), saponins (6) and others, have not been supported by convincing experimental and clinical evidence. Proteins appear to elicit the hypocholesterolemic response, mainly by activating liver LDL receptors (7,8), a mechanism tentatively attributed to specific protein components, i.e., the 7S globulin and its α-α' subunits (9).

As of now, there is clear evidence of the following: *1*) a hypocholesterolemic activity of isoflavone-free soy concentrates in humans (10); *2*) the inactivity of isoflavones in LDL receptor regulation in vitro (8) and in hypercholesterolemia in vivo, when given as pure substances (11,12); *3*) the apparent loss of activity of soy proteins after ethanol treatment for isoflavone removal (13), but also the inactivity of the ethanol-extracted material when added to a prototype hypercholesterolemic protein, i.e., casein (14). An essentially identical hypocholesterolemic response was reported recently in patients who consumed soy-based diets that included foods with a wide range of isoflavone concentrations (15).

To evaluate possible associations between the variable responses to soy protein intake in the different clinical studies in this field (16), we investigated through a proteomic approach the major available soy preparations used in almost all clinical studies by our group (17,18) and also in the majority of U.S.-based studies, at both the experimental and clinical levels (13,19).

Proteomics investigates the "absence vs. presence" in varying amounts of individual protein components in a test sample under different conditions. The protocol of choice for the analysis of such complex protein mixtures as whole homogenates or extracts is by two-dimensional electrophoresis (2-DE),[3] i.e., sequential migration along orthogonal directions in isoelectric focusing and SDS-PAGE. Microanalytical procedures based on mass spectrometry (MS) permit the identification of the proteins corresponding to the resolved spots. Protein identification requires "expected to found" comparisons, with reference to established databases. Matching is feasible on the basis of either identity or similarity; a parametrical

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[3] Abbreviations used: 2-DE, two-dimensional electrophoresis; CBB, Coomassie Brilliant Blue; Ig, immunoglobulin; IPG, immobilized pH gradient; MALDI, matrix-assisted laser desorption/ionization; MS, mass spectrometry; TPAA, total concentration polyacrylamide.

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scoring rates individual hits according to their statistical significance. Sequencing of complete genomes and proteomic projects are developing at a somewhat slower pace for plant species than for animals or unicellular organisms. The EU *Arabidopsis* Sequencing Project is currently carrying out an in-depth investigation on this model species. For soybeans, a total of 1150 entries are listed by ExPASy databases (261 in SWISS-PROT and 889 in TrEMBL), to be compared with 34064 (1663 in SWISS-PROT) for *Arabidopsis* and 40730 (8321 in SWISS-PROT) for *Homo sapiens*. Perspectives on the use of proteomics for genetic and physiologic studies in plants have been discussed in (20).

MATERIALS AND METHODS

Soy samples. The following samples were examined:

1. Total soybean seed proteins, extracted from soybean defatted flour with 50 mmol/L Tris/HCl pH 8.5 containing 200 mmol/L NaCl and 0.02% 2-mercaptoethanol. 7S and 11S globulins were purified by salting out with ammonium sulfate and by isoelectric precipitation (21,22).
2. Soy concentrates used in essentially all clinical studies in hypercholesterolemic patients in Italy, under the commercial names of Cholsoy/Croksoy (17,18). These concentrates are prepared in edible form after a patented heat treatment procedure and are free of isoflavones (10). These concentrates have a protein content of ~70%.
3. Soy protein isolates (SUPRO). Two different preparations were examined. Their protein concentrations were 65–75 g/100 g by Bradford analysis (23), whereas the concentration of nitrogen-containing compounds was 82.3 g/100 g as determined by carbon-hydrogen-nitrogen analysis.
4. Isoflavone-poor SUPRO, identical to 3 but treated with ethanol, thus providing a very low content of isoflavones (extracted SUPRO). Two preparations of these also were examined.

The SUPRO products were kindly donated by Dr. Susan Potter, Director of Research, PTI International, St. Louis, MO.

Quantification of isoflavones. Soybean proteins (100 mg) were suspended in a solution prepared with 1.2 mL acetonitrile, 0.2 mL of 0.1 mol/L HCl and 0.6 mL distilled water and stirred at room temperature for 2 h (24). After filtration on a paper filter, the solvent was evaporated in vacuum at a temperature <30°C. The residue was then redissolved in 0.1 mL methanol/water (80:20, v/v), and analyzed by HPLC. The analyses were conducted on a Hewlett-Packard quaternary pump (HP-1050), equipped with a Rheodyne injection and a Diode Array Detector (Hewlett-Packard, Palo Alto, CA). A 3D-Chemstation (Dos series) was used for data managing and calculations. Elution was monitored from 220 to 600 nm; the data at 254 nm were used for quantification. The elution proceeded with solvents A (0.1% acetic acid in water) and B (0.1% acetic acid in acetonitrile) at a gradient from 15% B to 35% B in 50 min, then isocratic for 10 min; the flow rate was 1 mL min (25). The minimum detectable amount was 0.005 mg/g.

Electrophoresis. 1-DE (SDS-PAGE) was run on 7.5–17.5% T polyacrylamide gradients in the discontinuous buffer system of Laemmli (26). 2-DE maps were obtained by immobilized pH gradient (IPG)-DALT (27,28). Proteins were separated by charge on a nonlinear pH 4–10 IPG (29) in the presence of 8 mol/L urea, then according to size by SDS-PAGE on 7.5–17.5% or 10–23% polyacrylamide gradients. Gels were stained with 3 g/L Coomassie.

Immunodetection. After SDS-PAGE, proteins were electroblotted onto a nitrocellulose membrane (30). Immunodetection was with a polyclonal *anti*-7S antibody (1:2,000 dilution) followed by a horseradish peroxidase anti-immunoglobulin (Ig)G antibody (1:2,000) and enhanced chemiluminescence zymography. The *anti*-7S antibody was obtained in our laboratory by boosting New Zealand White rabbits with purified antigen in complete Freund's adjuvant; the immune IgG was purified from the antiserum by ammonium sulfate precipitation according to standard protocols (31).

Preparation of samples for mass spectrometry. In-gel digestion with trypsin was performed according to published methods (32–34) modified for use with a robotic digestion system (Genomic Solutions, Huntington, UK). Silver-stained gel pieces were decolorized by washing with 30 μL of 15 mmol/L potassium ferricyanide/50 mmol/L sodium thiosulfate (35), followed by three washes with 100 μL deionized water. Cysteine residues were reduced with dithiothreitol and derivatized by treatment with iodoacetamide. The gel pieces were then dehydrated with acetonitrile and dried at 60°C before the addition of modified trypsin (Promega, Madison, WI; 10 μL at 6.5 mg/L in 25 mmol/L ammonium hydrogen carbonate). After incubation at 37°C for 8 h, the products were sequentially extracted with 25 mmol/L ammonium hydrogen carbonate, 5% formic acid and acetonitrile. Lyophilized extracts were redissolved in 0.1% formic acid before matrix-assisted laser desorption ionization (MALDI) and electrospray ionization MS/MS analysis.

MALDI mass spectrometry. MALDI mass spectra were recorded with a TofSpec 2E spectrometer (Micromass, Manchester, UK), equipped with a 337 nm nitrogen laser. The instrument was operated in the positive ion reflectron mode at 20 kV accelerating voltage with time-lag focusing enabled. The matrix was a mixture of α-cyano-4-hydroxy-cinnamic acid and nitrocellulose, applied to the target as a microcrystalline thin film by a modification of the procedure of Vorm et al. (36). Aliquots (0.75 μL) of the digest solution were deposited on the matrix film, allowed to dry and desalted by brief washing with 4 μL of ice-cold 0.1% trifluoroacetic acid. Spectra were internally calibrated using trypsin autolysis products, and the resulting peptide mass fingerprints were searched against a local copy of the nonredundant database maintained by the National Center for Biotechnology Information (37) using the Protein Probe search engine (Micromass). Where necessary, additional searches were performed using the program Mascot (38). An initial mass tolerance of 50 μg/g was used in all searches.

RESULTS

Determination of isoflavones. The four SUPRO preparations were analyzed to assess the range of isoflavone concentrations. The nonethanol-extracted products had the expected high isoflavone concentrations, albeit with some variability among the samples (**Table 1**), in line with differences reported in the literature for the raw seeds (39). Both ethanol-extracted batches had very low isoflavone concentrations with an unexpectedly large variability. For example, there was a 35-fold difference in daidzein concentration between batches with high and low isoflavone concentrations.

Identification of major components of Croksoy. **Figure 1** compares by 2-DE the whole soy flour extract vs. Croksoy concentrate (*panels A* and *B*). The major soy globulins and their subfractions were very clearly separated in soy flour (*panel A*). The major components resolved from the whole extract correspond to the subunits of soybean storage proteins, i.e., α, α' and β subunits from 7S globulin (β-conglycinin), heavy (H) and light (L) subunits from 11S globulin (glycinin). In Croksoy (*panel B*), few definite components were resolved in

TABLE 1

Isoflavone concentrations of different SUPRO preparations

	Unextracted		Ethanol-extracted		
	(M32-406)	(C5C-XPC001)	(M32-318)	(P7F-XRP)	Croksoy
			μg/g		
Daidzein	639	751	2.5	82.2	52.2
Glycitein	96	163	7.9	14.5	ND
Genistein	1587	2825	36.5	239	trace

[1] ND, not detectable.

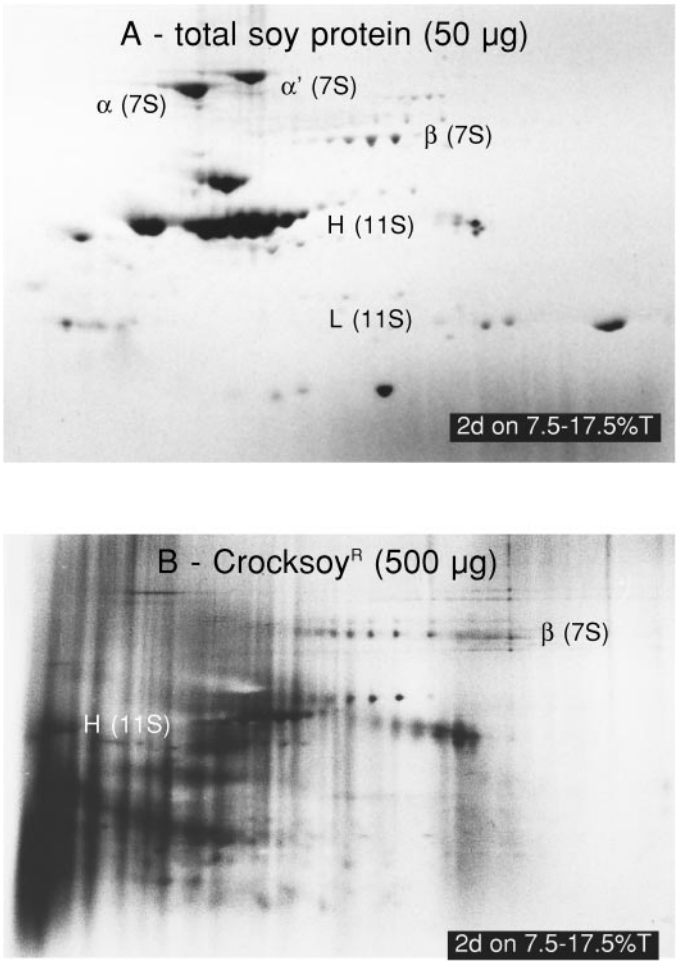

FIGURE 1 Two-dimensional electrophoresis (2-DE) of 50 μg total soy protein (*A*) and of 500 μg Croksoy protein (*B*). The position of the major globulin subunits is marked. Proteins were focused on a nonlinear 4–10 immobilized pH gradient (IPG), then fractionated by size on 7.5–17.5%T PAA gradients.

the presence of a blurred background; the $\alpha+\alpha$' subunits of 7S were not detected. The total stained material accounted for only a small percentage of the loaded sample (compare Fig. 1*B*, corresponding to 500 μg Croksoy protein, to 1*A*, 50 μg protein as total flour extract). The presence of intact H chains of 11S globulin was confirmed by MS (courtesy of Dr. P. Haynes, Washington University, Seattle).

Identification of major components of the SUPRO products. Similar to Croksoy, SUPRO products were characterized by a low staining yield (**Fig. 2**; 800 μg per sample). Unlike the total extract, the patterns of isolated soy proteins lacked all high molecular mass species and most stained spots were ≤25 kDa. Compared with the unextracted isolated SUPRO proteins (Fig. 2*A*), those that were alcohol-extracted (Fig. 2*B*) appeared enriched in some components.

Because there was no evidence of higher molecular weight species in any SUPRO preparations, we decided to evaluate by MALDI MS the identity of a number of randomly selected lower molecular weight spots. The major spots resolved by 2-DE (Fig. 2*C*) were excised, the protein digested with trypsin and the size of the resulting peptides analyzed (**Table 2**). Most of the spots corresponded to subunits of 11S globulin or to their fragments. Accordingly, SUPRO protein components may have resulted from degradation of higher molecular weight species.

To identify a possible residual trace of the 7S globulin, a detailed investigation was carried out by immunoblotting using an *anti*-7S antibody. **Figure 3** compares the migration in SDS-PAGE of a mixture of 7S and 11S globulin (lanes A–B) isolated soy proteins (lanes C–E). For the purified proteins, the antibody detected bands with the molecular mass of intact subunits (lane B), whereas in the isolated protein lane, most of the stained components were found at positions corresponding

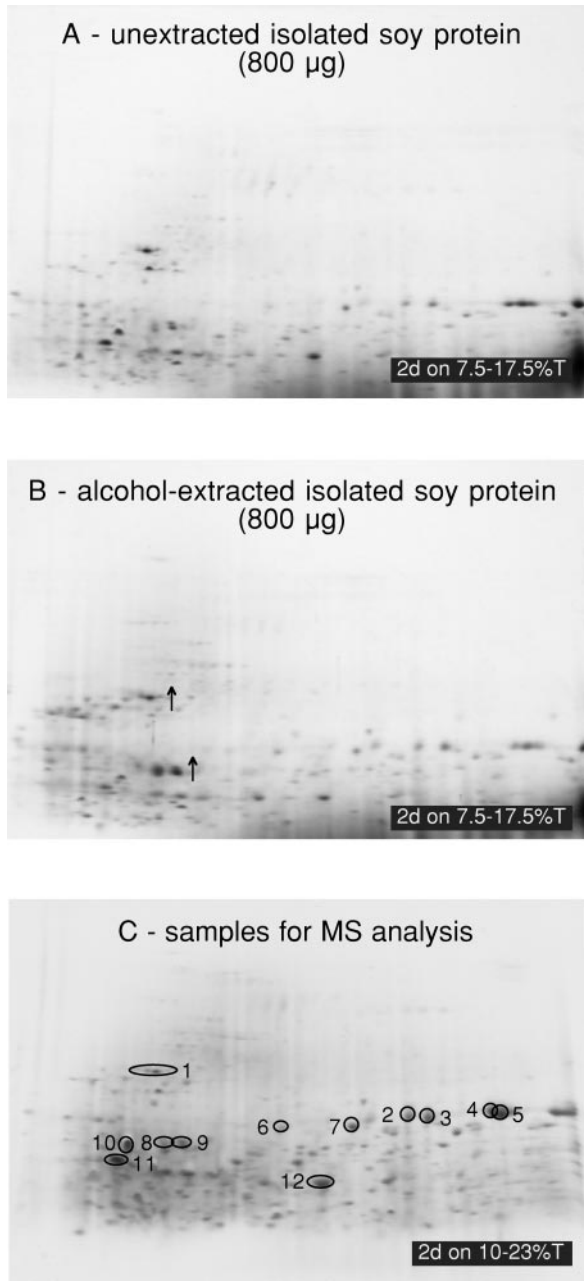

FIGURE 2 Two-dimensional electrophoresis (2-DE) of 8000 μg unextracted isolated soy protein (*A*) and of 800 μg alcohol-extracted isolated soy protein (*B*). Spots whose abundance is increased in alcohol-extracted vs. unextracted sample are marked with an arrow (↑). (*C*) In the samples for analysis by mass spectrometry, spots excised for protein identification are circled and marked with sequential numbers. Experimental procedures were as in Figure 1 except for (C) in which SDS-PAGE was run on a 10–20%T PAA gradient.

TABLE 2

Identification of the major spots resolved by two-dimensional electrophoresis (2-DE) of isolated soy proteins

Spot #	Protein
1	GLC2_SOYBN; GLCB_SOYBN; GLCA_SOYBN
2	GLC2_SOYBN (C-TERM FRAGMENT)
3	GLC1_SOYBN (C-TERM FRAGMENT)
4	GLC2_SOYBN (C-TERM FRAGMENT)
5	GLC1_SOYBN (C-TERM FRAGMENT)
6	GLC1_SOYBN (N-TERM FRAGMENT)
7	GLC1_SOYBN
8	ITRA_SOYBN; GLC1_SOYBN
9	ITRA_SOYBN; GLC1_SOYBN
10	ITRA_SOYBN: Glycinin alaBx
11	Glycinin AlaBx
12	Glycinin G4

to a size smaller than native 7S subunits (lane D vs. lane C). Once again, the immunoreactive material in isolated soy protein may have accounted for only a percentage of the loaded sample (compare lanes B and D). At very high sample loads (100 μg isolated soy protein in lane E), a distinct immunoreactivity was revealed in a gel region corresponding to very low molecular masses ($\leq$10 kDa).

DISCUSSION

Proteomic studies of plant specimens are most often carried out for the purpose of identification of species and cultivars in plants such as maize (40), barley (41) and durum wheat (42). The 2-DE methodological approach has never been applied to the evaluation of food proteins that have undergone a range of industrial processes. After an extensive search of the Chemical Abstracts, we did not find any references specifically related to proteomic studies on edible proteins. In addition to improving our knowledge of the protein composition of soybean products, we were seeking an explanation for the variability of cholesterolemic response to the different soy-based diets in clinical studies (16).

Evaluation of crude soy flour by the 2-DE gel method (Fig. 1) agreed well with our own observations and those of others using standard 1-DE SDS-PAGE gels (43). The 2-DE gels allow an improved separation of the 7S and 11S subfractions, with more complete characterization. This methodology was used to detect the absence of the α' subunit in the soy variant Keburi, which apparently does not regulate the LDL receptor (44).

Proteomic investigation of the Croksoy concentrate, which is the most widely used product in clinical studies in Italy and Switzerland and provides the clearest demonstration of the cholesterol-lowering activity of soy proteins (17,18), is compatible with the extensive degradation of 7S globulin (Fig. 1*B*) that results in immunoreactive products of size <25 kDa (44). 2-DE gels of Croksoy demonstrate the durability of the 11S globulin and subfractions thereof, apparently intact after the defatting and heat treatment procedures.

In the two different types of SUPRO products examined, ethanol- and nonethanol-treated, the isoflavone concentrations were remarkably variable (Table 1). For example, the two ethanol-treated products had a 35-fold difference in daidzein concentration. This difference is too great to be due only to the differences in the isoflavone contents of the raw seeds (39) and suggests the need for better standardization of the industrial procedures for isoflavone extraction.

The 2-DE gels of SUPRO products were rather surprising for two reasons. First, after standard loading of the protein, a large part of the material did not produce visible spots, suggesting that it was below precipitation and staining limits in size. This discrepancy was much greater than what was expected from the protein content of the preparations (g/100 g), evaluated by the Bradford method (23). Second, there appeared to be essentially no material with molecular weight >25kDa. These findings would suggest an essentially complete degradation of the 7S globulin by the industrial process, with a predominance of very low molecular weight material migrating in the gel with the salt front.

Identification of the predominant protein components of SUPRO (both ethanol- and nonethanol-treated) was carried out by MALDI MS. Most of the spots analyzed corresponded to fragmentation products of 11S globulin. To verify that SUPRO was not derived from any novel soy varieties, some of which are essentially devoid of the 7S globulin (45), an immunodetection process with a specific 7S antibody was carried out to assess the presence or absence of 7S fragmentation products. This investigation clearly showed that the industrial processes involved in SUPRO preparation preferentially degrade the 7S fraction, whereas they maintain to some extent the low molecular weight components (corresponding mainly to 11S subunits). Information in a recent U.S. patent indicated more marked cholesterol- and also triglyceride-lowering effects in animals fed a 7S-enriched, isoflavone-poor, soy product vs. a standard soy isolate (46).

A comparison of 2-DE gels from ethanol- and nonethanol-treated SUPRO indicated some potentially important protein changes. In particular (Fig. 2), some proteins appeared to have accumulated more in the ethanol-treated sample, possibly indicating a differential solubility of some of the protein com-

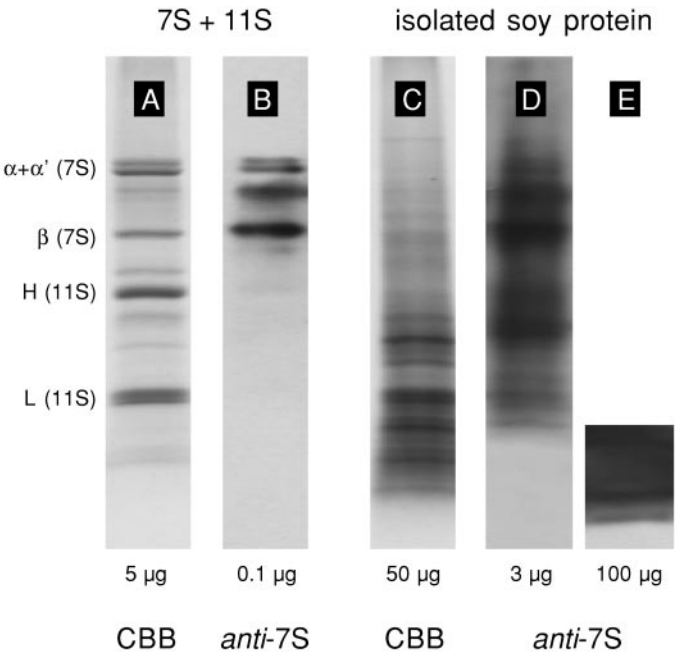

FIGURE 3 SDS-PAGE of 7S + 11S (lanes A–B) and of isolated soy protein (SUPRO, lanes C–E) is shown. Coomassie stain, lanes A (5 μg) and C (50 μg); immunodetection with *anti*-7S antiserum, lanes B (0.1 μg), D (3 μg) and E (100 μg, cropped image). Experimental procedures took place under reducing conditions on a 7.5–17.5%T PAA gradient.

ponents. In-depth identification of these changes in the lower molecular weight peptides warrants further study.

Detailed evaluation of dietary proteins has become of major interest now that peptides themselves appear to exert direct effects on major cardiovascular risk markers such as blood pressure (47) or cholesterolemia (48). Recent evidence indicates that the 7S α' subunit in particular may be beneficially modified by insertion of vasoactive peptides, thus leading to a directly hypotensive dietary protein (49). The structurally complex chemical nature of 7S globulin allows its stepwise dissociation into subunits; among these, the 7S α' in particular may allow release of active peptide components into the circulation. Initial testing of custom-designed peptides that regulate cholesterolemia by activation of LDL receptors has been reported (9).

Striking differences were noted between the Croksoy product, used in most studies describing LDL cholesterol reduction in excess of 20% in hypercholesterolemic patients (16–18), and the SUPRO product, which is widely distributed in North America but shown to be only moderately effective in patients (13). In fact, SUPRO appears highly fragmented, with a predominance of very low molecular weight peptides; the majority of the stainable components are derived from the 11S globulin, which is putatively less active in cholesterolemia (9). However, a recent patent describes a low phytate soy protein product, containing mainly 11S globulin and with significant hypocholesterolemic activity in rats (50). It could be confirmed by immunodetection, however, that the SUPRO product does contain the 7S globulin (Fig. 3).

A clear limitation of this study is that it provides only visual assessment of proteomic differences, and no direct comparisons have been made among the different protein preparations, such as in the management of severely hypercholesterolemic individuals. The observations of proteomic differences between ethanol-treated and untreated products may provide experimental support to the very recent hypothesis that an interaction between isoflavones and proteins may be lost due to ethanol treatment (5), which might explain why the loss of the cholesterolemic response in primates is not restored by readdition of the ethanol wash material.

Without knowing the industrial processes used for preparing SUPRO, one cannot argue how and why the 7S globulin is so sensitive to the treatment, whose purpose is it to prepare more edible, palatable and better digestible nutrients. The present study indicates, however, that there is considerable variability in the isoflavone concentrations of both ethanol- and nonethanol-treated SUPRO. Furthermore, better standardization of the products, which would include identifying the major protein/peptide components, would yield more effective dietary nutrients for hypercholesterolemia.

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