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Cloning, yeast expression, purification and biological activity of a truncated form of the soybean 7S globulin α′ subunit involved in Hep G2 cell cholesterol homeostasis☆

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Abstract

A truncated form of α′ chain (tα′), the soybean 7S globulin subunit previously demonstrated to be active in controlling the cholesterol and triglyceride homeostasis in *in vitro* and *in vivo* models, was cloned and expressed in the yeast *Pichia pastoris*. The recombinant polypeptide spanned 216 amino acid residues from the N-terminal side and included the N-terminal extension region of the soybean subunit. The tα′ polypeptide was purified by conventional biochemical techniques, and its potential to modulate the activity of low-density lipoprotein (LDL) receptor was evaluated in a human hepatoma cell line (Hep G2) by monitoring the uptake and degradation of labeled LDL. The LDL uptake (+192%) and degradation (+143%) by cells tested at the highest tα′ dose (8 μM) were similar to those found in cells incubated with 1 μM simvastatin, a potent inhibitor of cholesterol biosynthesis. The cell response to tα′ was found to be dose dependent. The use of a recombinant polypeptide ruled out the involvement of any other soybean component.

These findings open new prospects in the studies aimed at identifying soybean regulatory (poly)peptide(s) and the mechanism involved in this biological response, as a gateway to their utilization for the management of human health.

Keywords: 7S Soybean proteins; Truncated α′; Chain; Hep G2 cells; LDL Receptors; Cell cholesterol homeostasis

1. Introduction

The role of dietary soybean proteins in the control of lipidemic levels of hypercholesterolemic patients is a widely accepted issue [1]. In previous studies [2–4], the direct involvement of one subunit of the soybean 7S globulin, the α′ subunit, in the up-regulation of the low-density lipoprotein (LDL) receptor (LDL-R) was demonstrated in *in vitro* and *in vivo* systems, suggesting that biologically active (poly) peptides, capable of modulating cholesterol homeostasis, are likely to be produced by cell enzyme processing.

The native 7S globulin is composed of three randomly assorted polypeptide chains, the α′, α and β subunits [5], encoded by different genes. The mature α′ (accession no. P11827, UniProtKB/Swiss-Prot database) and α chains (accession no. P13916, UniProtKB/Swiss-Prot database) share an extended N-terminal region of about 140 amino acid residues which is missing from the β subunit (accession no. P25974, UniProtKB/Swiss-Prot database). Based on the peculiar amino acid sequence of the α′ extension region, this subunit was purified by metal affinity chromatography and orally administered to hypercholesterolemic rats, thus allowing to show both its plasma lipid-lowering properties and up-regulation of liver β-VLDL receptors [4]. On the other hand, since the molecular weight of the α′ subunit is around 71 kDa [6], it seems unlikely that it may cross *in vivo* the intestinal barrier with no modification. For this reason, our research has been directed to seeking the amino acid sequence(s) of α′ subunit responsible for the pharmacological effect. Since the core regions of the three subunits have more similar amino acid sequences, it is conceivable that the biological activity should reside in one or more (poly)peptides of the extension region. In principle, the localized but significant amino acid differences between the extension regions of the α′ and α chains would limit the number of peptides responsible for the biological activity. From these statements, our strategy was to test the effectiveness of both polypeptides with a hydrolyzed isoflavone-free soybean concentrate [7–8] and synthetic peptides having amino acid sequences that differed between the 7S soybean globulin subunits on the LDL-R modulation by Hep G2 cells. The results of these studies pointed out that a marked LDL-R up-regulation could be induced in HepG2 cells exposed to partially

Abbreviation: FCS, fetal calf serum; LDH, lactate dehydrogenase; LDL-R, LDL receptor; LPDS, lipoprotein-deficient serum; MEM, minimum essential medium; MTT, methyltetrazolium salt; PCR, polymerase chain reaction; tα′, truncated form of 7S soybean globulin α′ subunit.

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hydrolyzed polypeptides ranging from 3000 to 20,000 Da, as well as to a small 7S soybean synthetic peptide (2271 Da) at a concentration of 10^{-4} M [9]. These data suggested a potentially promising area for the development of dietary peptides characterized by important pharmacological properties. On the other hand, since the study obtained with small peptides is still currently under investigation and has not been conclusive so far [10], we decided to develop a different strategy, aimed at identifying an active limited region of the α′ chain.

To this purpose, the heterologous expression of a truncated form of the α′ chain, which will be referred to as tα′ in this article, was undertaken. The objective was achieved in secretion-competent cells of the yeast *Pichia pastoris*. The recombinant polypeptide was purified and its biological activity assessed in HepG2 cells. By the use of this biotechnological approach, adequate amounts of the recombinant polypeptide could be obtained and tested in *in vitro* trials, as a first step to further *in vivo* assays.

2. Materials and methods

2.1. Yeast, bacterial strains and chemicals

Pichia pastoris X33 (wild-type) strain (Invitrogen, San Diego, CA, USA) was used for yeast expression. The bacterial strain utilized for genetic manipulations was *E. coli* XL1-Blue (Invitrogen). Restriction enzymes *Pst*I and *Xba*I were purchased from Roche (Indianapolis, IN, USA) and *Sac*I from Fermentas (Ontario, Canada). Taq DNA polymerase was purchased from Invitrogen. Zeocin was purchased from Invivogen (San Diego, CA, USA). The oligonucleotides for PCR were obtained from Primm (Milan, Italy). Peptone, tryptone, yeast extract and agar were purchased from Becton Dickinson (Sparks, MD, USA).

Other chemicals were reagent grade from Sigma (St. Louis, MO, USA).

2.2. Media and growth conditions

Pichia pastoris X33 strain was cultured in yeast peptone dextrose (YPD) complete medium (2% peptone, 1% yeast extract, 2% glucose). The Mut^+ transformants were selected in plates containing YPD, agar (1.5%), 100 mg/ml zeocin. All yeast cultures were maintained at 30°C. For protein production, selected Zeo^r-Mut^+ transformants were cultured to an optical density of 5 at 600 nm in yeast peptone sorbitol (YPS) medium (2% peptone, 1% yeast extract, 2% sorbitol). Then methanol was added to 1% final concentration. All bacterial transformants were selected in plates of low-salt Luria-Bertani (LB) medium containing zeocin (1% tryptone, 0.5% yeast extract, 0.5% sodium chloride, 1.5% agar, 25 mg/ml zeocin). All bacterial cultures were maintained at 37°C.

2.3. Construction of tα′ gene expression vector

The tα′ gene was amplified by PCR on an expression *E. coli* plasmid DNA template (pT7-7) containing the sequence of interest (tα′). The oligonucleotide PIC-1 (5′-GAAGGAGATATACATG**CTGCAG**TGGAGGAAG-3′) was designed to generate a *Pst*I restriction site at the 5′ end of the tα′ gene. This mutation resulted in an alanine residue insertion in the N-terminal position of tα′. A second oligonucleotide PIC-2 (5′-CGATGC**TCTAGA**TCATCAGTTAAGGATAACGATG-3′) was designed to generate an *Xba*I restriction site at the 3′ end of the tα′ gene. Both oligonucleotides were dissolved in MilliQ (Waters, Milan, Italy) sterile water. The PCR mixture consisted of 0.5 mM primers, 0.8 mM dNTPs (Eppendorf, Hamburg, Germany), 30 ng of template (pT7-7/tα′), 2.5 U Taq DNA polymerase, PCR buffer (final composition: 50 mM KCl, 1.5 mM $MgCl_2$, 20 mM Tris-Cl (pH 8.4) and sterile water to a final volume of 25 mL). PCR amplification was carried out on a Perkin Elmer Geneamp PCR System 2400 thermocycler (Perkin Elmer, Wellesley, MA, USA) using the following conditions: initial denaturation (start): 97°C for 6 min; 30 cycles at 97°C for 1 min, 50°C for 1.5 min, 72°C for 1.5 min; final extension: 72°C for 10 min and maintained at 4°C. The PCR 650-bp product was cloned into the pPICZaB vector resulting in the pPICZaB-tα′ construct and transformed in XL1-Blue *E. coli* cells. Positive clones were selected on semisolid LB media containing tetracycline and zeocin. One of these clones was sequenced by Primm (Milan, Italy) to ensure that no mutation occurred in the pPICZaB-tα′ construct sequence. Thirty-five micrograms of the pPICZaB-tα′ construct and 30 μg of the expression vector pPICZaB (negative control) were linearized by digestion with the restriction enzyme *Sac*I and then purified.

2.4. Transformation of pPICZaB-tα′ in P. pastoris genome

Wild-type yeast cells were transformed by electroporation with 35 μg of linearized pPICZaB-tα′ construct and 30 μg of linearized pPICZaB on an Eppendorf (Hamburg, Germany) electroporator 2510 apparatus set at 1.5 kV. Transformants were first selected by plating on YPD plates containing 100 mg/mL of zeocin. In order to verify the integration of the construct into transformed *P. pastoris* genome, the Dneasy Plant Mini Kit (QIAGEN, Hilden, Germany) was used to extract genomic DNA. Genomic DNA was used as template to verify the insertion of the construct at the alcohol oxidase promoter (AOX1) site by PCR using 5′AOX1 (5′-GACTGGTTCCAATTGACAAGC-3′) and 3′AOX1 primers (5′-GCAAATGGCATTCTGACATCC-3′). The PCR mixture consisted of 0.5 mM primers, 0.25 mM dNTPs, 100 ng of genomic template and 30 ng of plasmidial template, 2.5 U Taq DNA polymerase, PCR buffer (final composition as above). PCR was performed using the following conditions: initial denaturation (start): 94°C for 10 min; 30 cycles at 94°C for 40 s, 60°C for 40 s, 72°C for 20 s; final extension: 72°C for 10 min and maintained at 4°C.

2.5. Expression and purification of tα′

Eighteen Zeo^+ transformants and one transformant containing pPICZaB expression vector (negative control) were grown in 50 mL of YPS medium at 30°C in a shaking (180 rpm) incubator to an OD_{600} of 5. The inducing phase was triggered by adding methanol to 1% final concentration and prolonged for 24 h. Aliquots of the supernatants were examined for expression of tα′ by SDS-PAGE. The clone transformant with the highest tα′ expression was selected for large-scale production of the recombinant protein and grown under the same conditions as above. About 1 L of culture was centrifuged at 11,300×*g* and 24°C for 15 min with a J2-21 M/E centrifuge (Beckman Instrument, Palo Alto, CA, USA). The proteins contained in the supernatant were precipitated by 70% ammonium sulfate and centrifuged at 11,300×*g* at 4°C for 30 min. The pellet was dissolved in 50 mL sterile water and precipitated by 100% acetone (1:1 v/v) at −30°C. The solution was centrifuged for 1 h as above. The pellet was dissolved in 25 mL 50 mM Tris-HCl (pH 7.4) and loaded on a DEAE-cellulose column (2.5×10 cm, Whatman, Maidstone, UK) equilibrated with the same buffer. The elution of retained proteins was carried out with the same buffer containing 0.15 and 0.25 M NaCl, respectively. The fraction eluted with 0.25 M NaCl displayed the greatest content of tα′.

2.6. Electrophoretic techniques

SDS-PAGE under reducing conditions (2% β-mercaptoethanol) was carried out on 12% polyacrylamide gels [11] using a mini-gel kit (Protean II cell, Bio-Rad). The gels were stained with Coomassie blue.

2.7. Cell cultures

The established human hepatoma cell line (HepG2) was obtained from American Type Culture Collection (Rockville, MD, USA). Eagle's Minimum Essential Medium (MEM), fetal calf serum (FCS), trypsin-EDTA (1×), penicillin (10^5 U/L), streptomycin (100 g/L), tricine buffer (1 mmol/L, pH 7.4) and nonessential amino acid solutions (100×) were from GIBCO (Madison, WI, USA). Petri dishes were from COSTAR (Cambridge, MA, USA). Filters were from Millipore (Bedford, MA, USA). The Protein Coomassie Plus Protein Assay kit was purchased from Pierce (Rockford, IL, USA). ^{125}Iodine, carrier free, in 100 mmol/L NaOH, was from Perkin Elmer Life Sciences (Boston, MA, USA). Sephadex G25 columns (PD10) were from Pharmacia Biotech (Uppsala, Sweden). LDH and the methyltetrazolium salt (MTT) kit were from Sigma Diagnostics (Milan, Italy). All other chemicals were of analytical grade from Merck (Darmstadt, Germany). Cells were grown in monolayers in 90-mm-diameter Petri dishes and maintained at 37°C in a humidified atmosphere of 95% air–5% CO_2 in MEM supplemented with 10% FCS, nonessential amino acid solution (1%, v/v), penicillin (10^5 U/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). For experiments designed to evaluate the LDL-R modulation, cells were seeded in 35-mm plastic dishes (3–5×10^5 cells) and used just before reaching confluence. In all cell culture experiments, the medium was changed every 2–3 days. In order to assess cell viability, culture media from cells exposed to tα′ at different concentrations were tested by MTT assay, essentially as described in Ref. [9]. Cell enzyme leakage was determined by measuring lactate dehydrogenase (LDH) activity, using a kinetic (LDH/LD) diagnostic kit (Sigma Diagnostics). LDL ($1.019 \leq d \leq 1.063$ g/L) was isolated by sequential preparative ultracentrifugation [12] from the plasma of clinically healthy normolipidemic volunteers. Lipoproteins were labeled according to the method of McFarlane as modified by Bilheimer et al. [13] and previously described [3]. ^{125}I-LDL was sterilized by filtration (Millipore filters, 0.45 μm pore size) and stored at 4°C until use. Human lipoprotein-deficient serum (LPDS) was prepared as previously described [9].

2.8. Uptake and degradation of ^{125}I-LDL

Monolayers of cells were preincubated at 37°C for 24 h in MEM supplemented with 5 g/100 g LPDS to up-regulate the LDL-Rs [2], in the presence/absence of tα′ at different concentrations listed in Fig. 1 or 3.5 μmol/L α′ purified soybean subunit or 1.0 μmol/L simvastatin. A fixed concentration (7.5 mg/L) of ^{125}I-LDL was then added to the medium and the incubation continued for a further 5 h at 37°C. Specific uptake (binding+internalization) and degradation of ^{125}I-LDL were evaluated as previously reported [2].

2.9. Statistical analyses

Differences in cell uptake and degradation of LDL after cell incubation with tα′ at different concentrations were determined by ANOVA followed by Dunnett's

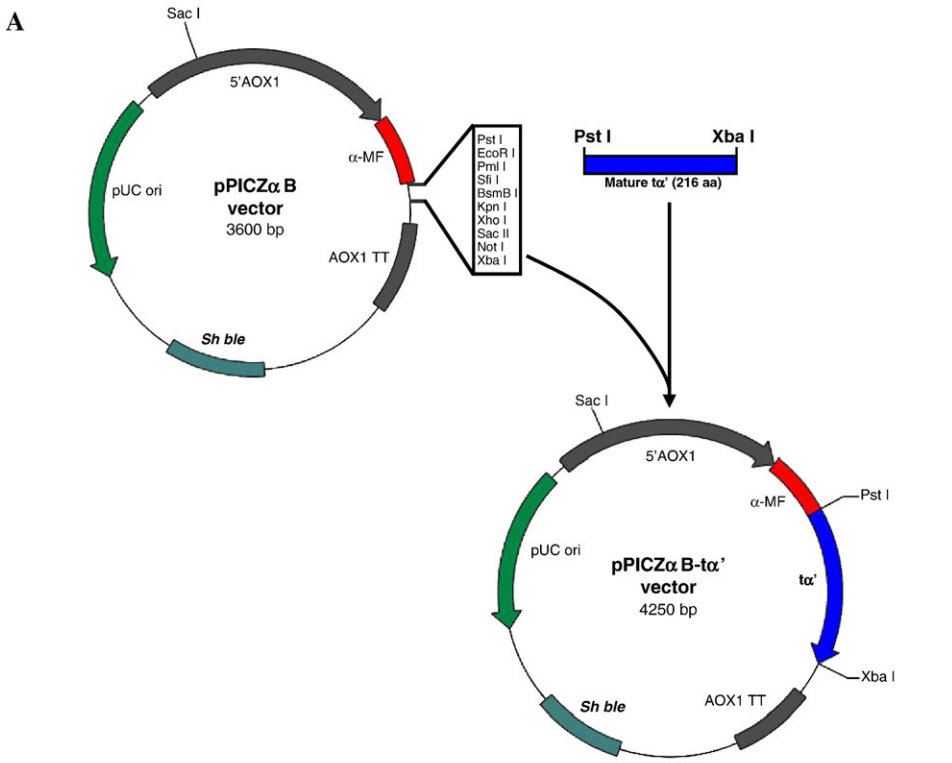

Fig. 1. (A) Overview of the pPICZαB-tα′ construct. Expression of tα′ is driven by the alcohol oxidase (AOX) methanol-inducible promoter (5′AOX1); the α-mating factor (α-MF) promotes secretion of the recombinant protein to the medium; AOX1 TT: AOX transcription termination region. The *Sh ble* gene confers resistance to zeocin; pUC Ori: origin of replication for the high-copy-number plasmid in *E. coli*. The other abbreviations refer to the cleavage positions of restriction enzymes; bp: base pair. (B) Deduced amino acid sequence of the recombinant polypeptide (tα′).

test. Values are expressed as means±S.D.; *P* values <.05 were considered as statistically significant.

3. Results

3.1. Expression of tα′ in P. pastoris

The structure of the plasmid used to transform *P. pastoris* cells and the deduced amino acid sequence from the cloned α′ fragment subunit are shown in Fig. 1A and B, respectively. As mentioned in Materials and methods, the only difference between the recombinant and wild-type polypeptides consisted in the N-terminal first amino acid residue which, for technical reasons, was an alanine in the recombinant chain. The clone showing the greatest production of recombinant polypeptide, as judged by SDS-PAGE analysis of the culture medium (not shown), was selected for large-scale production. Fig. 2 shows the electrophoretic analysis of the amplicons generated by PCR, to verify the integration of the construct into the plasmidial and yeast genomes. The size of the observed bands corresponded to that of the related genes in the respective samples, thus indicating that the insert is actually present in the transformed cells.

3.2. Purification of tα′

The purification of tα′ was achieved by a combination of precipitation steps and chromatographic approaches. Samples from each step were collected and analysed by SDS-PAGE (Fig. 3). Lanes 1 and 2 of Fig. 3 (Panel A) show the electrophoretic patterns of the yeast clones transformed with the empty and the integrated construct, respectively. As it is shown, a faint band at 26 kDa, indicating the

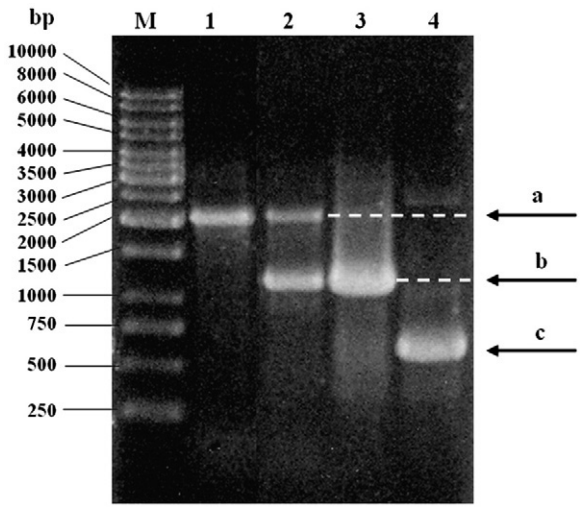

Fig. 2. Agarose electrophoretic gel of transformed *P. pastoris* amplicons. Lane M: 1-kb DNA ladder marker; Lane 1: genomic DNA wild-type X33 strain; Lane 2: genomic DNA clone X33-pPICZαB-tα′; Lane 3: plasmidial DNA pPICZαB-tα′; Lane 4: plasmidial DNA pPICZαB (empty construct). Arrow a: AOX1 gene; arrow b: amplicon including tα′ recombinant gene; arrow c: amplicon including the empty construct.

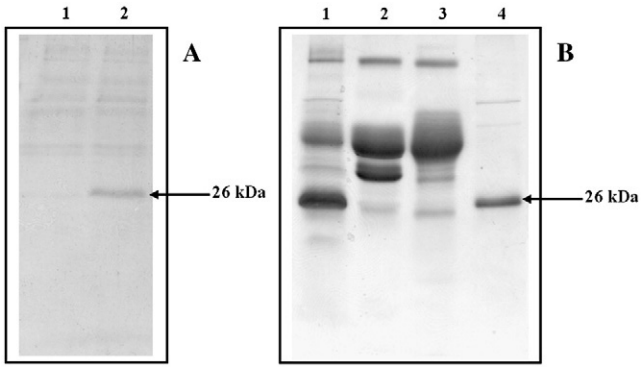

Fig. 3. SDS-PAGE under reducing conditions of *P. pastoris* culture media (A) and tα′ purification steps (B). (A) Lane 1: X33-pPICZαB (empty construct) induced with 1% of methanol; Lane 2: X33-pPICZαB-tα′ induced with 1% of methanol. (B) Lane 1: Acetone powder; Lane 2: DEAE-cellulose unbound fraction; Lane 3: DEAE-cellulose 150 mM NaCl eluted fraction; Lane 4: DEAE-cellulose 250 mM NaCl eluted fraction.

expression of the recombinant polypeptide, was detected in the positive clone only. N-Terminal amino acid sequence analysis of this band corresponded to the first five amino acids of the deduced sequence, thus confirming that the cloned 26-kDa polypeptide corresponded to the tα′ chain.

The effect of the purification steps on the homogeneity of the identified polypeptide is shown in Fig. 3 (Panel B). A relevant enrichment of the polypeptide was already achieved with the precipitation procedures, but further chromatographic steps removed the main contaminant proteins and allowed the recovery of the recombinant polypeptide in an almost homogenous form. The purity of this sample was judged to be suitable for the cell assays.

3.3. Biological activity of tα′

The addition of tα′ and the purified α′ soybean subunit, as a positive control, to HepG2 cells produced a significant rise in LDL-R-mediated uptake and degradation compared to the untreated cells (Fig. 4). The LDL modulation by tα′ was dose dependent and greater

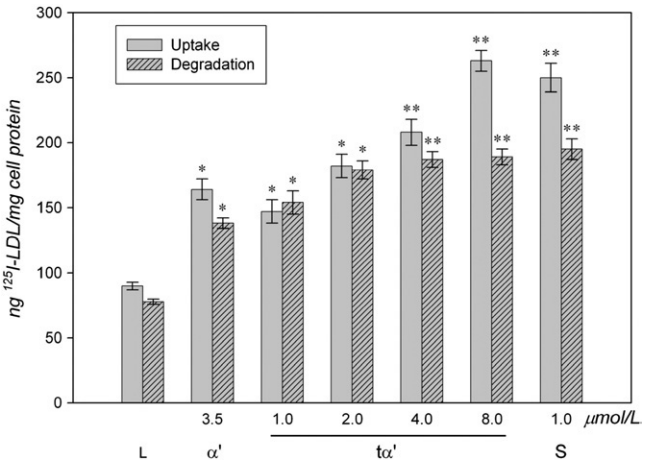

Fig. 4. Effect of tα′ and full-length α′ soybean subunit on the LDL uptake and degradation by HepG2 cells. Confluent monolayers of HepG2 cells were preincubated at 37°C for 24 h in MEM with 5% LPDS, in the presence of purified α′ subunit (α′), the recombinant polypeptide (tα′) or simvastatin (S) at the concentrations indicated in the figure. L: LPDS as the control. After the addition of ^{125}I-LDL, cells were incubated for an additional 5 h and then analyzed as described in Materials and methods. The data are means±S.D. of three independent experiments, each performed in quadruplicate. *P<.05 *vs.* LPDS and **P<.001 *vs.* LPDS.

than that recorded in cells incubated with an almost identical molar concentration of the purified α′ subunit, i.e., +131% vs. +82% uptake and +140% vs. +77% degradation, respectively. The result obtained with tα′ at the highest concentration (8 μM) was similar to that of the second positive control, 1 μM simvastatin, i.e., +192% vs. +172% uptake and +143% vs. +150%, respectively. At no tα′ concentration was there any evidence of cellular toxicity, as determined by the MTT and LDH assays (not shown).

4. Discussion

The cholesterol- and triglyceride-lowering capacity of soybean proteins has been demonstrated clearly. Soybean protein oral administration is currently the most potent dietary tool for treating hypercholesterolemic patients, thus providing a unique opportunity for the management of adults and very young subjects. Moreover, it is clearly established that plasma cholesterol reduction is greater in patients having a high baseline degree of cholesterolemia [14].

The hypothesis that proteins *per se* reduce blood cholesterol arose from experimental studies indicating that a shift from animal to plant proteins in the diet activates the LDL-R system in the liver of laboratory animals [15], as well as in circulating lymphomonocytes of hypercholesterolemic patients [16]. To identify the soybean protein components responsible for the cholesterol-lowering effect, *in vitro* studies were carried out with a human hepatoma cell line that is highly sensitive to factors regulating LDL-R expression and cholesterol biosynthesis/breakdown. We concluded that the purified α′ subunit from the 7S soybean globulin up-regulated LDL-Rs in Hep G2 cells [3] and confirmed this finding in cholesterol-fed rats [4]. Although these data support the hypothesis that the protein moiety is responsible for the observed biological effect, arguments may be raised in favor of α′ chain *in vivo* metabolic fate, since peptides and amino acids are normally produced by the action of gastric and/or intestinal proteolytic enzymes. However, an increasing number of animal and plant (poly)peptides are being claimed to play relevant regulatory functions, often attributed to anti-oxidant, antiproliferative and anti-inflammatory effects [17]. As far as soybean is concerned, experimental evidence clearly indicates the possibility that peptides and even small compact proteins, such as the Bowman–Birk inhibitor, may be adsorbed [18], thus eliciting a number of effects, including anticancer, anti-inflammatory, radioprotective ones [19]. Other peptides have been shown to exert hypotensive effects [20]. Recently, an LDL-R transcription stimulating peptide (FVVNATSN), deriving from the 7S globulin β chain, has been identified from a soybean hydrolysate prepared by a protease from *Bacillus amyloliquefaciens* and then by chemical synthesis [21]. In this case, an increased LDL-R transcription (+148%) was detected in Hep G2 cells exposed to the peptide at a concentration of 100 μM. Other peptides arising from the 11 S globulin have been shown to exert similar but lower activity [21].

Therefore, in the search for a small-sized polypeptide, putatively responsible for the observed biological activity, we described in the present work the cloning, yeast expression and purification of a recombinant polypeptide which contained the N-terminal extension region of the soybean α′ subunit and proved to be even more effective on LDL uptake and degradation than the full-length α′ chain. The present results suggest that the amino acid sequence capable of inducing the biological response lies in the N-terminal extension domain of α′ chain. Moreover, we found that the truncated polypeptide exerts its effects at concentrations in the order of magnitude as those of simvastatin, a potent hypolipidemic drug. How this effect could be achieved *in vivo* is difficult to establish as yet and this will require further investigations. However, *in vitro* interaction between the 7S soybean globulin and thioredoxin, a small multifunctional protein with a redox-active disulfide-dithiol in

the conserved active site sequence Cys-Gly-Pro-Cys, has been shown [3]. This finding might explain the longer lag phase of LDL oxidation induced by cupric oxide observed in rabbits fed cholesterol-rich diet containing soybean protein *vs.* that found in rabbits fed the same diet but containing casein as protein source [22].

The data obtained in the present study are intriguing because they show for the first time that a truncated recombinant form of soybean 7S globulin α′chain is active in an *in vitro* model at concentrations (<10 μM) that are similar to those reported for simvastatin. Moreover, the use of a recombinant protein rules out any involvement of other protein and nonprotein soybean components, including isoflavones, for which clear benefits have not been claimed [23].

Studies are in progress to identify the minimally active region(s), by means of further reducing the size of the truncated form, and to trace the metabolic pathway of the properly labeled polypeptide in target cells. Both approaches are aimed at the elucidation of the mechanism (s) underlying the cell lipid homeostasis. In addition, in order to reveal the potential lipid-lowering properties of the recombinant polypeptide, experiments will be performed in rats fed a cholesterol-rich diet, an animal model of human hypercholesterolemia.

The results of these studies could lead to the development of functional foods with beneficial effects on various diseases, including hyperlipidemia and cardiovascular disease, to be used alone or in combination with drugs in lipid-lowering therapies.

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