

Heterologous expression and purification of the soybean 7S globulin α' subunit extension region: *In vitro* evidence of its involvement in cell cholesterol homeostasis

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ABSTRACT

In a previous paper, the biological activity of a 216-amino acid recombinant truncated form of the soybean 7S globulin α' subunit, known to control cholesterol and triglyceride homeostasis, was described. In this work, a shorter version of the polypeptide chain, spanning 142 amino acid residues from the N-terminus and thus exclusively including the so-called extension region, was cloned and over-expressed in *Pichia pastoris*. The yield of the recombinant polypeptide, which was termed $\alpha'E$, was 8-fold greater than the previous truncated version. The $\alpha'E$ polypeptide was purified by simple conventional biochemical techniques to make it available for biological assays. Human hepatoma cell lines (Hep G2) were used to monitor the uptake and degradation of labeled low-density lipoproteins (LDL), according to an established procedure. The LDL uptake (+86%) and degradation (+94%) by cells tested at the highest $\alpha'E$ dose (2 μ M) were similar to those found in cells incubated with 1 μ M simvastatin, a potent inhibitor of cholesterol biosynthesis. Additionally, the cell response to $\alpha'E$ was found to be dose-dependent. The present findings strongly suggest that this recombinant polypeptide, or a fragment thereof, is the molecular determinant for cholesterol homeostasis and open new prospects for understanding the mechanism involved in this biological response, as a gateway to its utilization in lipid-lowering therapies.

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Introduction

The role of dietary soybean proteins in the control of lipidemic levels of hypercholesterolemic subjects is widely accepted [1]. Despite intense studies in this area, the molecular determinant/s of the observed effects has/ve not been unequivocally identified. Nonetheless, soybean 7S globulin, the so called β -conglycinin, has received more attention in this respect, because it was shown to play a role in the up-regulation of liver high-affinity LDL receptors [2]. This protein was also shown to reduce plasma triglycerides in humans [3] and hyperlipidemic rat models [4]. From the structural viewpoint, the native 7S globulin is a randomly assorted hetero-trimer of α' , α , and β subunits [5]. In previous studies [6–8], the direct involvement of one subunit of the soybean 7S globulin (the α' chain) in the up-regulation of the LDL-receptor was demonstrated by *in vitro* and *in vivo* model systems, suggesting that biologically active peptides, capable of modulating cholesterol homeostasis,

are likely to be produced by cell enzyme processing. The mature α' chain (UniProtKB/Swiss-Prot database accession No. P11827) consists of an extended N-terminal region of 141 amino acid residues and a core region of 436 amino acids (Fig. 1). The core region is highly homologous to the same region of the other 7S globulin subunits, namely the α and β subunits (UniProtKB/Swiss-Prot database accession Nos. P13916 and P25974, respectively). Interestingly, the incubation of Hep G2 cells with the α or β subunits was ineffective on the LDL-receptor activation [6]. The extension region is present only in the α' and α chains. The two extension regions have similar but not identical amino acid sequences, which have allowed the preparative separation of the α' chain and the assessment of its biological activity [8]. However, due to the difficulties of preparing homogenous α' subunit in amounts suitable for biological assays and in order to help identification of the bioactive region, the heterologous expression of a truncated form of this chain was undertaken [9]. In that paper, the expression and purification of an α' polypeptide consisting of 216 amino acid residues from the N-terminus and, thus, containing the cited extension region plus a stretch of the core region (Fig. 1), allowed us to test the biological activity of this polypeptide.

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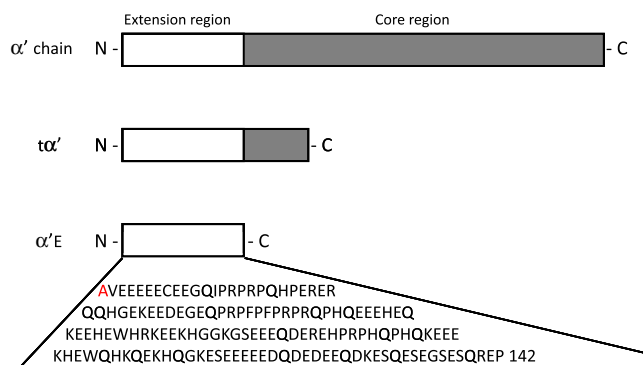


Fig. 1. Outline of soybean 7S globulin α' chain and its recombinant derivative polypeptides, namely truncated α' (α') and α' extension ($\alpha'E$) region. Amino acid sequence of this latter chain is reported. The alanine residue in red has been added to the wild-type sequence for cloning strategy.

Indeed, the LDL uptake and degradation induced by the truncated polypeptide on Hep G2 cells was similar to that measured on the entire α' subunit. Moreover, the results obtained were comparable to those obtained with simvastatin, a powerful cholesterol-lowering drug. The relevance of that study, carried out on a recombinant protein, was that it showed the activity was due to a soybean protein and not to any other potentially bioactive compound, such as isoflavones. However, the expression yield was relatively low, as well as the recovery and purification of the polypeptide, and further studies or applications of this recombinant polypeptide were intrinsically difficult. For this reason and with the aim of identifying a shorter bioactive sequence responsible for the activity, in the present work we undertook cloning, expression, purification and biological assay of a soybean recombinant polypeptide consisting of the sole extension region. This polypeptide will be referred to as " α' extension" ($\alpha'E$)¹ in this paper.

The results on the biological activity of this new polypeptide open new perspectives for the development of dietary proteins associated to relevant pharmacological properties in the area of plasma lipid homeostasis control.

Materials and methods

Yeast, bacterial strains and chemicals

Pichia pastoris X33 (WT) strain and pPICZ α B plasmid (Invitrogen, San Diego, CA) were used for yeast expression. The bacterial strain utilized for genetic manipulations was *Escherichia coli* XL1-Blue (Invitrogen, San Diego, CA). Restriction enzymes PstI and XbaI were purchased from Roche (Indianapolis, IN), SacI from Fermentas (Ontario, Canada), Taq DNA polymerase was purchased from Invitrogen (San Diego, CA), zeocin was purchased from Invitrogen (San Diego, CA). The oligonucleotides for PCR were obtained from Primm (Milano, Italy). Peptone, tryptone, yeast extract and agar were purchased from Becton Dickinson and Company (Sparks, MD).

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

Media and growth conditions

P. pastoris X33 strain was cultured in YPD (Yeast Peptone Dextrose) complete medium (2% peptone, 1% yeast extract, 2%

glucose). The Mut⁺ transformants were selected in plates containing YPD, agar 1.5%, 100 μ g/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 Buffered Yeast Peptone Sorbitol (BYPs) medium consisting of 2% peptone, 1% yeast extract, 2% sorbitol, 13.2 mM K₂HPO₄, 86.8 mM KH₂PO₄, pH 7.5. Then methanol was added to 1% final concentration. All bacterial transformants were selected in plates of low-salt LB (Luria-Bertani) medium containing zeocin (1% tryptone, 0.5% yeast extract, 0.5% sodium chloride, 1.5% agar, 25 μ g/mL zeocin). All bacterial cultures were maintained at 37 °C.

Construction of $\alpha'E$ gene expression vector

The $\alpha'E$ gene was amplified by PCR on the expression *P. pastoris* plasmid DNA template (pPICZ α B- α') used in a previous paper [9] and containing the sequence of interest ($\alpha'E$). The forward oligonucleotide primer (5'-GAAAAGATAGATTAAAGCTGCAGTGGAGGAAG-3') was designed to generate PstI restriction site at the 5' end of $\alpha'E$ gene. This mutation resulted in the insertion of an alanine residue as N-terminal amino acid. A second reverse oligonucleotide primer (5'-CCCTTCTATTCTTCTAGATCATGGTTCTCTTTGAGACTC-3') was designed to generate XbaI restriction site at the 3' end of the $\alpha'E$ gene. Both oligonucleotides were dissolved in milliQ (Waters Corp., Milano, Italy) sterile water. The PCR mixture consisted of 0.5 mM primers, 0.8 mM dNTPs (Eppendorf, Hamburg, Germany), 30 ng template (pPICZ α B- α'), 2.5 U Taq DNA Polymerase, PCR buffer (final composition: 50 mM KCl, 1.5 mM MgCl₂, 20 mM Tris-Cl, pH 8.4 and sterile water to a final volume of 25 μ L). PCR amplification was carried out on a Perkin Elmer Geneamp PCR System 2400 thermocycler (Perkin Elmer Corp., Wellesley, MA) using the following conditions: start at 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. The PCR 425 bp product was cloned into pPICZ α B vector resulting in the pPICZ α B- $\alpha'E$ construct and transformed in XL1-Blue *E. coli* cells. Positives clones were selected on semisolid LB media containing tetracycline and zeocin. One of these clones was sequenced by Primm (Milano, Italy) to ensure that no mutation occurred in pPICZ α B- $\alpha'E$ construct sequence. Twenty micrograms pPICZ α B- $\alpha'E$ construct was linearized by digestion with the restriction enzyme SacI and then purified.

Transformation of pPICZ α B- $\alpha'E$ in *P. pastoris* genome

Wild-type (wt) yeast cells were transformed by electroporation with 20 μ g linearized pPICZ α B- $\alpha'E$ construct and 30 μ g linearized pPICZ α B, as the negative control (already available in our laboratory), on an Eppendorf (Hamburg, Germany) electroporator 2510 apparatus set at 1.5 kV. Transformants were first selected by plating on YPD plates containing 100 μ g/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'-GCAATGGCATTCTGACATCC-3'). The PCR mixture consisted of 0.5 mM primers, 0.25 mM dNTPs, 100 ng of genomic template, 2.5 U Taq DNA Polymerase, PCR buffer (final composition as above). PCR was performed using the following conditions: start at 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.

¹ Abbreviations used: PCR, polymerase chain reaction; $\alpha'E$, α' extension region of 7S soybean globulin α' subunit; LDL-R, low density lipoprotein receptor; LPDS, lipoprotein-deficient serum; MEM, minimum essential medium; FCS, fetal calf serum; LDH, lactate dehydrogenase; MTT, methyltetrazolium salt.

Expression and purification of $\alpha'E$

Forty Zeo⁺ transformants and one transformant containing pPICZ α B expression vector as the negative control were grown in 50 mL of BYPS medium at 30 °C in a shaking (180 rpm) incubator to an OD₆₀₀ = 5. The inducing phase was triggered by adding methanol to 1% final concentration every 24 h and prolonged for 72 h. Aliquots of the supernatants were examined for expression of $\alpha'E$ by SDS–PAGE. The clone transformant with the highest $\alpha'E$ expression was selected for large-scale production of the recombinant protein and grown under the same conditions as above. About 1 L culture was centrifuged at 11,300 × g and 24 °C for 15 min with a J2-21 M/E, Beckman Instrument, Palo Alto, CA. The proteins contained in the supernatant were precipitated by 50–90% ammonium sulfate and centrifuged at 11,300 × g at 4 °C for 30 min. The pellet was dissolved in 200 mL sterile water and acetone was added to 75% final concentration at –30 °C, to recover the recombinant polypeptide. The solution was then centrifuged for 1 h as above. For further purification, the pellet was dissolved in 100 mL 50 mM Tris–HCl, pH 7.5 and loaded on a DEAE-cellulose column (6 × 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 $\alpha'E$.

Protein concentration assay

The concentration of $\alpha'E$ was assessed by optical absorbance at 280 nm by using the extinction coefficient of 0.633 for 1 mg/mL concentration, as calculated on the basis of the amino acid sequence through Prot-Param Tool at <http://www.expasy.ch/tools/protparam.html>.

Electrophoretic techniques

SDS–PAGE under reducing conditions (2% β -mercaptoethanol) was carried out on 12% polyacrylamide gels [10] by the mini-gel kit (Protein II cell, Bio-Rad). The gels were stained with Coomassie Blue.

Cell cultures

The established human hepatoma cell line (HepG2) was obtained from American Type Culture Collection (Rockville, MD). Eagle's minimum essential medium (MEM), fetal calf serum, trypsin–EDTA (1×), penicillin (10⁵ U/L), streptomycin (100 g/L), tricine buffer (1 mmol/L, pH 7.4) and non-essential amino acid solutions (100×) were from GIBCO (Madison, WI). Petri dishes were from COSTAR (Cambridge, MA). Filters were from Millipore (Bedford, MA). The Protein Coomassie Plus Protein Assay kit was purchased from Pierce (Rockford, IL, USA). ¹²⁵Iodine, carrier free, in 100 mmol/L NaOH, was from Perkin Elmer Life Sciences (Boston, MA). Sephadex G25 columns (PD10) were from Pharmacia Biotech (Uppsala, Sweden). LDH and MTT kit were from Sigma Diagnostics (Milano, 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% CO₂ in MEM supplemented with 10% fetal calf serum (FCS), non-essential amino acid solution (1%, v/v), penicillin (10⁵ U/L), streptomycin (0.1 g/L), tricine buffer (20 mmol/L, pH 7.4), NaHCO₃ (24 mmol/L) and sodium pyruvate (0.11 g/L). For experiments designed to evaluate the LDL receptor modulation, cells were seeded in 35 mm plastic dishes (3–5 × 10⁵ 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 the compounds under study ($\alpha'E$ at different concentrations, 3.5 μ M purified α' subunit, 2 μ M $\alpha't$ and, 1 μ M simvastatin) were tested by methyltetrazolium salts (MTT) assay, essentially as described in Ref. [11]. Cell enzyme leakage was determined by measuring lactate dehydrogenase (LDH) activity, using a kinetic (LDH/LD) diagnostic kit (Sigma Diagnostics). LDL (1.019 ≤ *d* ≤ 1.063 g/L) fraction 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 [7]. ¹²⁵I-LDL were sterilized by filtration (Millipore filters, 0.45 μ m pore size) and stored at 4 °C until use. Human lipoprotein deficient serum (LPDS) was prepared as previously described [11].

Uptake and degradation of ¹²⁵I-LDL

Monolayers of cells were preincubated at 37 °C for 24 h in MEM supplemented with 5 g/100 g LPDS. The LPDS amount used in the experiment induces a weak expression of the LDL-receptor pathway in Hep G2 cells, a prerequisite for evaluating the potential of different compounds to up-regulate the LDL-receptor [2]. Cells were incubated in the presence/absence of $\alpha'E$ at different concentrations listed in Fig. 4 or 3.5 μ mol/L α' purified soybean subunit or 2 μ mol/L $\alpha't$ or 1.0 μ mol/L simvastatin. A fixed concentration (7.5 mg/L) of ¹²⁵I-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 ¹²⁵I-LDL were evaluated as previously reported [2]. Control cells were incubated in MEM supplemented with 5 g/100 g LPDS in the absence of the compounds under study.

Statistical analyses

Differences in the LDL uptake and degradation of incubated cells with various $\alpha'E$ concentrations were determined by ANOVA followed by Dunnett's test. Values are expressed as means ± SD of three independent experiments, each performed in quadruplicate; *P* values <0.05 were considered as statistically significant.

Results

Expression of $\alpha'E$ in *P. pastoris*

The primary structures of the whole soybean 7S globulin α' chain, with the core and extension regions, and the previously cloned truncated α' chain are schematically shown in Fig. 1. The amino acid sequence of the $\alpha'E$ recombinant polypeptide generated in this work, consisting in the exclusive extension region of the original subunit, is also shown. As mentioned in the section 'Materials and methods', the only difference between the recombinant and wild-type polypeptides consisted in the N-terminal insertion of an amino acid residue which, for cloning technical reasons, was an alanine in the recombinant chain.

The clone showing the greatest production of the recombinant polypeptide, as judged by SDS–PAGE analysis of the culture medium, was selected for large-scale production (Fig. 2). In the same figure, the electrophoretic patterns of the culture media of the previously cloned truncated polypeptide, $\alpha't$ [9] and the empty construct are shown for comparison. While no major band was visible in the empty construct, a weak band of 26 kDa was present in the culture medium of the $\alpha't$. Conversely, the intensity of the novel recombinant $\alpha'E$ band at about 17 kDa was much stronger under the same expression conditions. Its apparent size fitted well

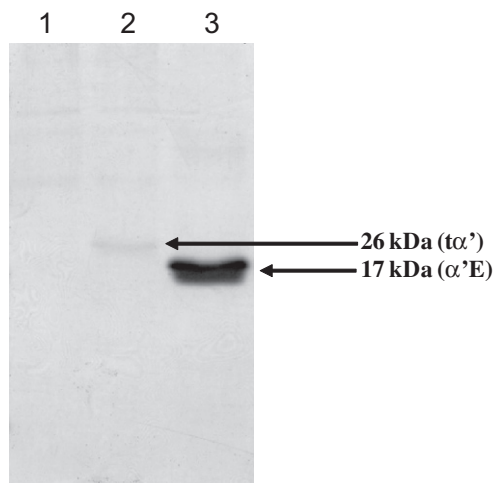


Fig. 2. SDS-PAGE under reducing conditions of *Pichia pastoris* culture media induced with 1% of methanol. Lane 1: X33-pPICZαB (empty construct); lane 2: X33-pPICZαB-α'E; lane 3: X33-pPICZαB-α'E.

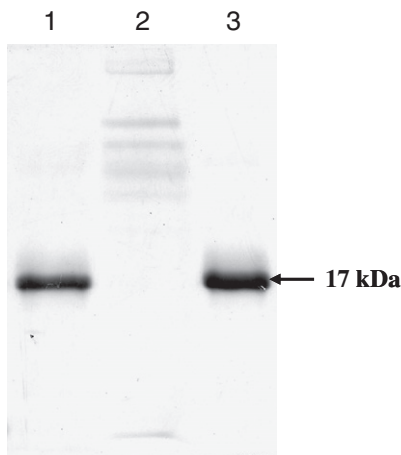


Fig. 3. SDS-PAGE under reducing conditions of α'E polypeptide purification. Lane 1: acetone powder of the culture medium; lane 2: DEAE-cellulose 150 mM NaCl eluted fraction; lane 3: DEAE-cellulose 250 mM NaCl eluted fraction.

with the mass calculated from the amino acid sequence, i.e. 17,385.9 Da.

Purification of α'E

The purification of α'E was achieved by a combination of precipitation steps and chromatographic approaches as described under the section 'Materials and methods'. Samples from each step were collected and analyzed by SDS-PAGE (Fig. 3). Lane 1 shows the electrophoretic pattern of α'E after the two precipitation steps with ammonium sulfate and acetone, respectively. As it can be seen, already with these simple and scalable steps, the polypeptide reaches a very high purification level. However, for further purification aimed at obtaining an extremely pure polypeptide for biological assays, an ion exchange chromatography step was carried out, as described under the section 'Materials and methods'. The 150 mM NaCl-eluted fraction did contain some high Mr polypeptide bands and no trace of α'E, while the fraction eluted with 250 mM NaCl consisted exclusively of the α'E polypeptide. The purity of this sample was judged to be well suited for the cell assays.

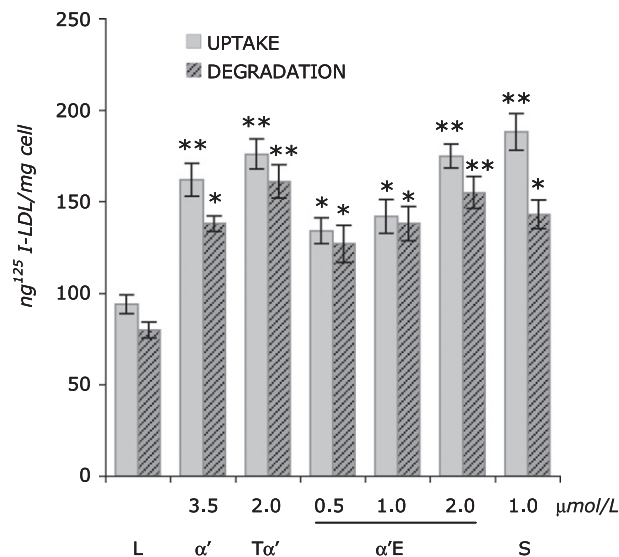


Fig. 4. Effect of α'E, α'E and full-length α' soybean subunit on LDL uptake and degradation by HepG2 cells. Confluent monolayers of HepG2 cells were preincubated at 37 °C for 24 h in MEM with 5% LPDS, in the presence of α' purified subunit (α'), the recombinant polypeptides α'E and α'E, or simvastatin (S) at the concentrations indicated in the figure. L: LPDS as the control. After the addition of ¹²⁵I-LDL, cells were incubated for an additional 4 h and then analyzed as described in the section 'Materials and methods'. The data are means ± SD of three independent experiments, each performed in quadruplicate. *P < 0.05 vs LPDS and **P < 0.001 vs LPDS.

After purification, the amount of α'E polypeptide recovered from 1 L culture medium was 71.1 mg. When this amount was compared with that previously obtained with the truncated α' form under identical conditions, i.e. 8.7 mg [9], an overall yield increase of about 8-fold could be calculated. Preliminary data on the yield obtained with a pilot fermentator indicated even greater yields, approaching 15-fold (not shown).

Biological activity of α'E

The treatment of HepG2 cells with α'E as well as with α'E and the intact α' soybean subunit, as positive controls, caused a significant rise in LDL receptor-mediated uptake and degradation compared to the untreated cells (Fig. 4). At no α'E concentration was there any evidence of cellular toxicity, as determined by the MTT and LDH assays (data not shown).

The LDL-R modulation by α'E was dose-dependent and at the highest concentration (2 μM) the LDL uptake and degradation was similar to that recorded in cells incubated with an almost identical molar concentration of α'E subunit (+86% vs +87% uptake and +94% vs +100% degradation, respectively) and, to the result obtained upon incubation with 1 μM simvastatin (uptake +100% and, degradation +78%, respectively). The data obtained in the present work on the shortened α' polypeptide chain confirmed the LDL-R up-regulation observed in previous works and indicated that the observed biological activity lies in a polypeptide of smaller size, as compared to the full length α' subunit, corresponding to the sole extension region of this soybean subunit.

Discussion

The cholesterol and triglyceride lowering capacity of orally administered soybean proteins is supported by both early [14,15] and recent [16] studies, as well as by the US health claim of 1999 [17]. The new frontiers of the research activities in the field are now understanding the mechanism of action underlying the biological effect and the identification of the bioactive region(s)

in the soybean 7S globulin polypeptide. The two aspects are mutually related each other.

In previous papers we first showed that the 7S globulin α' chain is capable of decreasing plasma lipid concentrations in rats fed on cholesterol-rich diet, as well as in suitable cellular models [8,7], respectively. Although these data supported the hypothesis that the whole polypeptide is responsible for the observed biological effect, arguments may be raised on α' chain *in vivo* metabolic fate, since peptides and amino acids are normally produced by the action of gastric and intestinal proteolytic enzymes. However, an increasing number of animal and plant (poly)peptides are being claimed to play relevant regulatory functions, often related to anti-oxidant, anti-proliferative and anti-inflammatory effects (for a review on the topic see Ref. [18]). More recently, a truncated form of the α' chain, roughly covering one third of the full-length polypeptide from the N-terminus, was shown to be biologically active in the same cellular model [9]. By adopting the same strategy, a shorter recombinant chain was expressed in yeast and proved to be active in this paper. This polypeptide chain exclusively consisted of the α' chain extension region, where a peculiar amino acid sequence is present (see Fig. 1). Indeed, several histidine residues potentially capable of binding metals, in particular Zn^{++} , and a number of proline residues often in crucial positions to block the action of digestive proteolytic enzymes, such as trypsin, are present in the sequence. Whether these features are related to the biological activity of the chain still has to be assessed.

The heterologous expression of the $\alpha'E$ region gave much better yield with respect to the previous version of the polypeptide (α'). No attempt to find out the molecular reason/s for that was undertaken. However, these results obtained under laboratory conditions promise even better yields when the fermentation is conducted in a fermentator under more effective and controlled growth conditions. Therefore $\alpha'E$ candidates as a potential end product for a development strategy of a lipid-lowering drug. Still our efforts in identifying the active minimal chain are continuing.

Recently, a 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 [19]. In this case, an increased LDL-R transcription (+148%) was detected in Hep G2 cells exposed to the peptide at a concentration of 100 μ M. Other peptides arising from the 11S globulin have been shown to exert similar activity [15]. The detection in this work of a similar activity displayed by the $\alpha'E$ region different sequence would allow us to hypothesize that not a specific amino acid sequence, rather a physico-chemical feature is responsible for the biological effect. Further investigations are therefore needed. These will be addressed to identify the minimally active region(s), by means of further reducing the size of the α' chain by biotechnological or chemical synthesis, and to trace the metabolic pathway of labeled promising polypeptide in cell systems. In addition, in order to reveal the potential lipid-lowering properties of the recombinant polypeptide *in vivo*, experiments will be performed in rats fed on cholesterol-rich diet as animal models of human hypercholesterolemia.

The results of these studies are the basis for 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.

Author disclosures

A. Consonni, M.R. Lovati, C. Manzoni, A. Parolari, P. Morazzoni, C. Magni, M. Duranti, no conflicts of interest.

References

- [1] C.R. Sirtori, M.R. Lovati, Soy protein and cardiovascular disease, *Curr. Atheroscler. Rep.* 3 (2001) 47–53.
- [2] M.R. Lovati, C. Manzoni, A. Corsini, A. Granata, R. Frattini, R. Fumagalli, C.R. Sirtori, Low-density receptor activity is modulated by soybean globulins in cell culture, *J. Nutr.* 122 (1992) 1971–1978.
- [3] H. Kambara, H. Motohiko, K. Takamatsu, M. Kito, Triglyceride-lowering effect of soybean β -conglycinin in humans, *Ther. Res.* 23 (2002) 85–89.
- [4] T. Aoyama, M. Kohono, T. Saito, K. Fukui, K. Takamatsu, T. Yamamoto, Y. Hashimoto, M. Hirotsuka, M. Kito, Reduction by phytate-reduced soybean β -conglycinin of plasma triglycerides level of young and adult rats, *Biosci. Biotechnol. Biochem.* 65 (2001) 1071–1075.
- [5] N. Maruyama, R. Sato, Y. Matsumura, H. Goto, E. Okuda, S. Nakagawa, S. Utsumi, Structure–physicochemical function relationships of soybean β -conglycinin constituent subunits, *J. Agric. Food Chem.* 47 (1999) 5278–5284.
- [6] M.R. Lovati, C. Manzoni, E. Gianazza, C.R. Sirtori, Soybean protein products as regulators of liver low-density lipoprotein receptors. I. Identification of active β -conglycinin subunits, *J. Agric. Food Chem.* 46 (1998) 2474–2480.
- [7] C. Manzoni, M. Duranti, I. Eberini, H. Scharnagl, W. Maerz, S. Castiglioni, M.R. Lovati, Subcellular localization of soybean 7S globulin in Hep G2 cells and LDL receptor up-regulation by its α' constituent subunit, *J. Nutr.* 133 (2003) 2149–2155.
- [8] M. Duranti, M.R. Lovati, V. Dani, A. Barbiroli, A. Scarafoni, S. Castiglioni, C. Ponzone, P. Morazzoni, The α' subunit from soybean 7S globulin lowers plasma lipids and upregulates liver β -VLDL receptors in rats fed a hypercholesterolemic diet, *J. Nutr.* 134 (2004) 1334–1339.
- [9] A. Consonni, M.R. Lovati, C. Manzoni, A. Pizzagalli, P. Morazzoni, M. Duranti, 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, *J. Nutr. Biochem.* 9 (2010) 887–891.
- [10] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature* 227 (1970) 680–685.
- [11] M.R. Lovati, C. Manzoni, E. Gianazza, A. Arnoldi, E. Kurowska, K.K. Carroll, C.R. Sirtori, Soy protein peptides regulate cholesterol homeostasis in Hep G2 cells, *J. Nutr.* 130 (2000) 2543–2549.
- [12] R.Y. Havel, H.A. Eder, J.H. Bradgon, The distribution of centrifugally separated lipoproteins in human serum, *J. Clin. Invest.* 34 (1955) 1345–1353.
- [13] D.W. Bilheimer, S. Eisenberg, R.I. Levy, The metabolism of very low density lipoprotein proteins. I. Preliminary in vitro and in vivo observations, *Biochim. Biophys. Acta* 260 (1972) 212–221.
- [14] M.R. Lovati, C. Manzoni, P. Agostinelli, S. Ciappellano, L. Mannucci, C.R. Sirtori, Studies on the mechanism of the cholesterol lowering activity of soy proteins, *Nutr. Metab. Cardiovasc. Dis.* 1 (1991) 18–24.
- [15] M.R. Lovati, C. Manzoni, A. Canavesi, M. Sirtori, V. Vaccarini, M. Marchi, G. Gaddi, C.R. Sirtori, Soybean protein diet increases low-density lipoprotein receptor activity in mononuclear cells from hypercholesterolemic patients, *J. Clin. Invest.* 80 (1987) 1498–1502.
- [16] C.R. Sirtori, I. Eberini, A. Arnoldi, Hypocholesterolaemic effects of soya proteins: results of recent studies are predictable from the Anderson meta-analysis data, *Br. J. Nutr.* 97 (2007) 816–822.
- [17] US Food and Drug Administration, Food labeling health claims: soybean protein and coronary heart disease. Final rule, *Fed. Regist.* 64 (1999) 57699–57733.
- [18] A. Scarafoni, C. Magni, M. Duranti, Molecular nutraceuticals as a mean to investigate the positive effects of legume seed proteins on human health, *Trends Food Sci. Technol.* 18 (2007) 454–463.
- [19] S.J. Cho, M.A. Juillerat, C.H. Lee, Identification of LDL-receptor transcription stimulating peptides from soybean hydrolysate in human hepatocytes, *J. Agric. Food Chem.* 56 (2008) 4372–4376.