Large-scale preparation of biologically active recombinant ovine obese protein (leptin)

Arieh Gertler[a,*], Julie Simmons[b], Duane H. Keisler[b]

[a] *Institute of Biochemistry, Food Science and Nutrition, Faculty of Agricultural, Food and Environmental Quality Sciences, The Hebrew University of Jerusalem, Rehovot 76100, Israel*
[b] *Animal Science Department, University of Missouri, Columbia, MO 65211, USA*

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Abstract Prokaryotic expression vector pMON3401 encoding full size A(-1) ovine leptin was prepared by polymerase chain reaction (PCR) of previously described cDNA. *E. coli* cells transformed with this vector overexpressed large amounts of ovine leptin upon induction with nalidixic acid. The expressed protein found in the inclusion bodies was refolded and purified to homogeneity on Q-Sepharose and SP-Sepharose columns, yielding two electrophoretically pure fractions (leptin-Q and leptin-SP), composed respectively of 90 and 95% of monomeric protein of the expected molecular mass of 16 kDa. The purified protein was capable of interacting with antibodies raised against GST-ovine leptin and to bind specifically to ventromedial hypothalamus of ewes. The biological activity of both fractions resulting from proper renaturation was further evidenced by their ability to stimulate DNA synthesis in leptin-sensitive BAF/3 cells transfected with a long form of human leptin receptor construct.

Key words: Obese protein; Leptin; Ovine; Recombinant

1. Introduction

Obese protein (OB), also known as leptin, serves as a protein signal secreted from adipose tissue and acts on a central nervous system that regulates ingestive behavior and energy balance [1]. Although the sequence of various leptins from 10 mammalian species was recently compiled and the three-dimensional structure of human leptin mutant W100E was recently elucidated [2], so far only two recombinant mammalian leptins (mouse and human) are available [3,4]. Recently a more efficient method for preparation of a renaturated recombinant human leptin overexpressed in *E. coli* was also reported [5]. Although direct comparison of its biological potency was not presented, the authors claim that the potency of this refolded protein is similar to that of leptin secreted by *E. coli* [3]. So far studies of leptin effect were mainly limited to rodents and the effect of this protein on metabolism and nutritional behavior of farm animals, which require large quantities of the recombinant leptin has not yet been investigated. To answer this challenge we describe in the present paper a highly efficient method for preparation of biologically active ovine leptin. Cloning of ovine leptin cDNA was recently described [6] and its sequence deposited in GenBank (number U84247). Ovine leptin has, respectively, 88% and 84% sequence identity to human and mouse leptins and differs by only two amino acids from bovine leptin [2,6].

*Corresponding author. Fax: (972) (89) 476189.
E-mail: gertler@agri.huji.ac.il

2. Materials and methods

2.1. Materials

Carrier-free $Na^{125}I$ and [^{3}H]thymidine were purchased from the New England Nuclear Corp. (Boston, MA, USA). Molecular weight (M_r) markers for gel electrophoresis, RPMI 1640 medium, nalidixic acid, were obtained from Sigma Chemical Co. (St. Louis, MO, USA). SDS-PAGE reagents were purchased from BioRad Laboratories (Richmond, CA, USA). Superdex75 HR 10/30 column, SP-Sepharose (fast flow) and Q-Sepharose (fast flow) were purchased from Pharmacia LKB Biotechnology AB (Uppsala, Sweden). All other chemicals were of analytical grade.

2.2. Construction of ovine leptin expression vector

The synthetic gene for leptin was constructed using polymerase chain reaction (PCR) technology, with a GeneAmpTM PCR Reagent Kit (Perkin Elmer, Norwalk, CT, USA). Synthetic oligonucleotides (primers) were used to generate a double-stranded DNA from a template, of GST-leptin (Simmons and Keisler, unpublished results) in addition to restriction enzyme sites for cloning. The forward primer 5′-GATATCGAATTCCCATGGCAGTGCCGATCCGCAAGGTCCAG encoded an *Nco*I restriction-enzyme site and an initiator methionine codon immediately upstream to alanine codon and the mature leptin. The reverse primer 5′-GGATCCCTGCAGAAGCTTTCAGCAGCCGGGACTCAGGTC encoded the *Hin*dIII restriction site and TAA termination codon immediately after the final codon (cysteine). PCR was performed using the Taq polymerase (20 cycles) and the PCR product was cleaned, digested with *Nco*I and *Hin*dIII restriction enzymes prior to ligation to parental vector pMON3401 using T4 DNA ligase. The ligation product was transfected to DHα5 *E. coli* cells, prior to transformation of MON105 cells [7]. Automatic DNA sequencing was performed to confirm the proper sequence.

2.3. Determination of the amino-terminal sequence

Automated Edman degradation technique was used to determine the amino-terminal protein sequence. Degradations were performed on an ABI Model 470A gas phase sequencer (Foster City, CA, USA) using the standard sequencing cycle [8]. The respective PTH-amino acid derivatives were identified by RP-HPLC analyses, using an ABI Model 120A PTH analyzer fitted with a Brownlee 2.1 mm i.d. PTH-C_{18} column.

2.4. Determination of purity and monomer content

SDS-PAGE was carried out according to Laemmli [9] using 15% gel. Gels were stained with Coomassie Brilliant Blue R. HPLC gel-filtration chromatography on a Superdex75 HR 10/30 column, using 25 mM Tris-HCl buffer, pH 8, containing 150 mM NaCl was performed on 200-μl aliquot of Q-Sepharose and SP-Sepharose columns-eluted fractions and freeze-dried samples dissolved in H_2O.

2.5. Binding experiments

Purified leptin-SP was iodinated by iodogen procedure according to the protocol described previously for iodination of growth hormone [10]. Slices of ovine hypothalamus were prepared as described previously [6] and the binding of iodinated leptin to the ventromedial hypothalamus of food-restricted ewes was assayed using a protocol described by Devos et al. [11].

2.6. Western analysis

The antiserum that we used to perform the Western was generated in our laboratory. It was produced in a rabbit to GST-ovine leptin

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which we expressed (Simmons and Keisler, unpublished). One hundred μg of GST-ovine leptin in 0.5 ml saline was emulsified with 0.5 ml of Freund's complete adjuvant. Three weeks later the rabbits were retreated with 100 μg of GST-ovine leptin in 0.5 ml of saline and 0.5 ml of Freund's incomplete adjuvant and after an additional week the antiserum was withdrawn. Standard SDS-PAGE and Western blot techniques were used to determine whether antiserum produced in a rabbit against GST-ovine leptin recognizes the two ovine leptin fractions. Briefly, samples were separated on 12.5% SDS-PAGE mini gels and electrophoretically transferred to nitrocellulose membranes. Membranes were incubated with polyclonal antisera to GST-ovine leptin. Proteins were visualized after incubation with anti-rabbit IgG alkaline phosphatase conjugated second antibody followed by nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate (Promega, MD, WI, USA).

2.7. BAF/3 proliferation bioassay

The bioassay was based on incorporation of ^{3}H-TdR into leptin-sensitive BAF/3 1442-Cl 4 cells transfected with long form of human leptin receptor according to a protocol described by Verploegen et al. [12]. Human and mouse leptins that were used as positive controls were prepared from the baculovirus infected cells [13].

3. Results and discussion

3.1. Expression, refolding and purification of ovine leptin

The sequence analysis of the cDNA revealed total identity to the published sequence indicating that the PCR preparation of the insert was correct. *E. coli* MON105 cells transformed with the expression plasmids containing leptin cDNA were incubated in 500 ml of Terrific Broth (TB) [14] medium by shaking at 200 rpm at 37°C in 3-l flasks to an A_{600} of 0.9, after which nalidixic acid (50 mg/flask) was added. The cells were incubated an additional 4 h, harvested by 5-min centrifugation at 10 000×*g* and frozen at −20°C. In a preliminary experiment performed with four clones the induced cells expressed leptin (∼16 kDa) as a main cell protein (Fig. 1A) and clone 011 was chosen for large-scale preparation. Over 95% of the leptin protein was found in the inclusion bodies (Fig. 1B) which were according to the detailed procedure used previously for preparation of bovine placental lactogen [15] with two additional washings with 1% Triton X-100. The inclusion-body pellet obtained from 5 flasks was solubilized in 150 ml of 4.5 M urea buffered with 10 mM Tris base. The pH was increased to 11.3 with NaOH, cysteine was added to 1 mM, the clear solution was gently stirred at 4°C for 6 h, diluted with 3 vols of 0.67 M L-arginine and dialyzed for 48 h against 5×10 l of 10 mM Tris-HCl, pH 8. The solution was then loaded at 120 ml/h onto a Q-Sepharose column (20 ml bead volume), pre-equilibrated with 10 mM Tris-HCl, pH 8.0 at 4°C. Over 80% of the monomeric leptin was not absorbed and was fully recovered in the breakthrough fraction (not shown). The column was subsequently washed with 0.05 and 0.5 M NaCl (Fig. 2A). As evidenced by gel-filtration chromatography on a Superdex75 column, the 0.05 M eluate contained a fraction composed mainly of monomeric leptin (over 90%) and dimers (∼10%). A small quantity of dimeric and oligomeric leptin was eluted with 0.5 M NaCl (Fig. 2A).

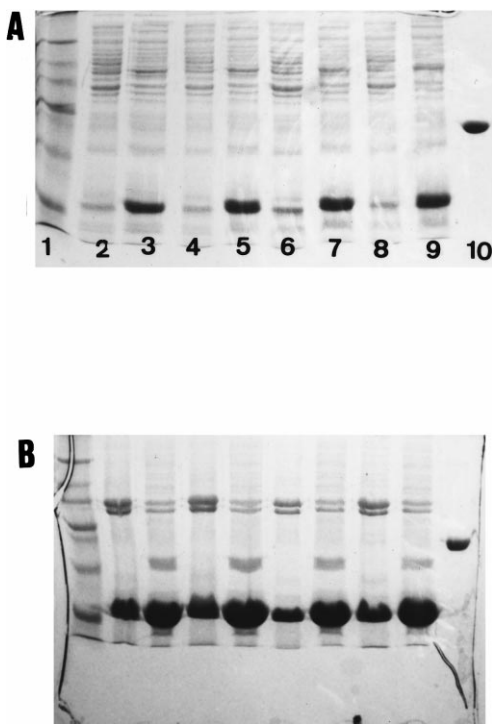

Fig. 1. SDS-PAGE analysis of the expression of ovine leptin in homogenate of 4 clones of MON 105 *E. coli* bacteria transformed with pMON3401 plasmid carrying leptin DNA (A) and in inclusion bodies prepared from the same clones (B). Lane 1: molecular mass markers (in kDa): 96, 66, 45, 35, 29, 24, 20 and 14.5; lanes 2, 4, 6, and 8: homogenates or inclusion bodies from four clones without induction; lanes 3, 5, 7 and 9: homogenates or inclusion bodies from the respective clones 4 h after induction with nalidixic acid. Lane 10: ovine placental lactogen (molecular mass 23 kDa). The lane numbers in B correspond to the same numbers in A.

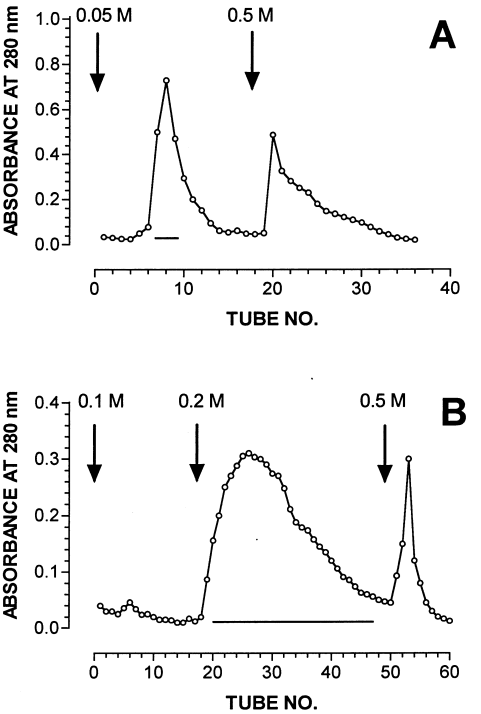

Fig. 2. Purification of inclusion bodies-extracted and refolded ovine leptin on a Q-Sepharose (A) and SP-Sepharose (B) column. The fraction containing inclusion body proteins solubilized in 4.5 M urea in 40 mM Tris-HCl at pH 11.3 and dialyzed against 10 mM Tris-HCl, pH 9, buffer (600 ml), was applied to the Q-Sepharose column. Fractions eluted from the Q-Sepharose with 0.05 M NaCl (see underlined peak) were pooled (leptin-Q). The non-absorbed eluate (breatkthrough fraction) was collected, adjusted to pH 5 with 10% HAc and applied to an SP-Sepharose column. Fractions eluted with 0.2 M NaCl (see underlined peak) were pooled (leptin-SP). For other details see text.

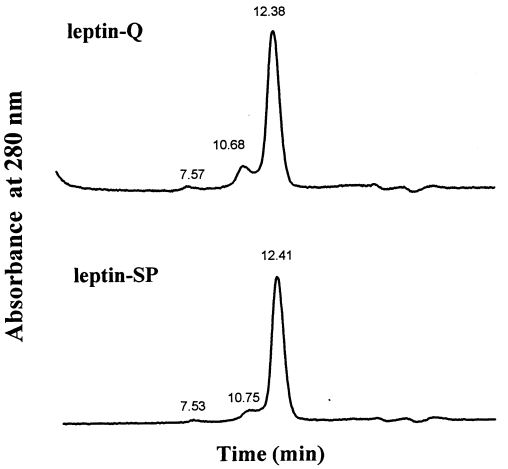

Fig. 3. Gel filtration of purified ovine leptin-Q and ovine leptin-SP on a Superdex 75 HR 10/30 column. Aliquots (200 ml) of lyophilized fractions (0.2 mg/ml) were applied to the column and the eluate was monitored by absorbance at 280 nm. The column was developed with 25 mM Tris-HCl buffer, pH 8, containing 150 mM NaCl at 1 ml/min and calibrated with bovine serum albumin (66 kDa, RT = 9.33 min), egg albumin (45 kDa, RT = 10.48 min), extracellular domain of hGH receptor (28 kDa, RT = 11.27 min) and ovine placental lactogen (23 kDa, RT = 11.72 min).

The breakthrough fraction from the Q-Sepharose column was adjusted to pH 5 with 10% HAc and applied to a SP-Sepharose column (1.2×8 cm) previously equilibrated with 25 mM NaAc buffer, pH 5. Elution was carried out using a discontinuous NaCl gradient in the same buffer at a rate of 90 ml/h, and 10-ml fractions were collected (Fig. 2B). The pure monomeric leptin (as evidenced by gel filtration on a Superdex column) was eluted as a wide peak with 200 mM NaCl. Leptin monomer-containing fractions from both columns (see Fig. 2 underlined) were dialysed against 0.1% $NaHCO_3$ and lyophilized. Inclusion bodies prepared from 2.5 l fermentation culture yielded ∼80 mg protein obtained from a Q-Sepharose column (leptin-Q) and ∼250 mg protein obtained from a SP-Sepharose column (Leptin-SP). As shown in Fig. 3, both fractions contained almost pure monomeric protein with RT = 12.38 and 12.41 min, respectively. Both fractions yielded only one band of ∼16 kDa on 15% SDS-PAGE performed in the presence or absence of reducing agent (not shown). The molar extinction coefficient of ovine leptin at 280 nm was calculated using the molar extinction coefficients of tyrosine (1197), yielding a value of 2394 M^{-1} cm^{-1}. Amino-terminal analysis of the first 6 amino acids yielded the expected sequence, namely AVPIRK in both fractions. Even though there was a methionine codon prior to alanine, it was almost completely cleaved by an amino peptidase [16]. Recent preparations using bacterial cultures grown in 10-l fermentor enabled us to prepare ∼0.5 g of leptin-Q and ∼2 g of leptin-SP from 10 l of bacterial culture (not shown).

3.2. Western analysis

Both Q- and SP-leptin fractions reacted with anti-GST-ovine leptin antibody which also recognizes human and porcine leptin (Keisler and Simmons, unpublished data). Only one band with a molecular mass of ∼16 kDa was observed (not shown). This band did not react with preimmune control sera.

3.3. Binding experiments

Incubation of ovine ^{125}I-leptin (SP) with slices of ovine hypothalamus (Fig. 4) clearly indicates specific binding to the ventromedial area (Fig. 4A). Total displacement of the ovine ^{125}I-leptin-SP was observed with addition of an excess of unlabeled leptin (Fig. 4B).

3.4. Biological activity in vitro

The biological activity of both leptin fractions was compared to that of human and mouse leptin (Fig. 5). The results were analyzed by PRIZMA software [17] according to non-linear regression sigmoidal dose-response curve. In all analyses the goodness of fit for the non-linear correlation was very high ($R^2 > 0.97$). Half of the maximal response (EC_{50}) in ovine-Q, ovine-SP, mouse and human leptins was achieved, respectively, at a 2.28×10^{-11}, 3.47×10^{-11}, 4.72×10^{-11} and 1.48×10^{-10} M concentration. The differences between the EC_{50} values of two ovine fractions and the mouse leptin were not statistically ($P > 0.05$) significant. The maximal response obtained with human leptin was slightly higher as compared to ovine or mouse leptins.

3.5. Conclusion

Two electrophoretically pure recombinant ovine leptin fractions (leptin-Q and leptin-SF) composed of over 90–95% of monomer and a small quantity of dimers were obtained. Since the N-terminal analysis of both fractions was identical we assume that they differ by small charge change. The more acidic fraction leptin-Q was likely created resulting by deamination of one or more glutamine residues during the refolding procedure at high pH. As shown above this difference had no effect on the biological activity which was equal to that of human and mouse leptins. The present method is sufficient for preparation of large quantities of biologically active re-

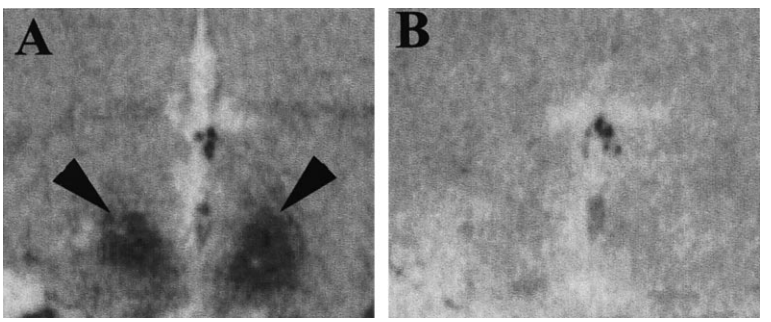

Fig. 4. Photomicrograph of a 12-μm section of frozen ovine hypothalamus incubated with ^{125}I-leptin indicating specific binding to the ventromedial area (A). Displacement of ^{125}I-leptin was observed with addition of an excess of unlabeled leptin (B).

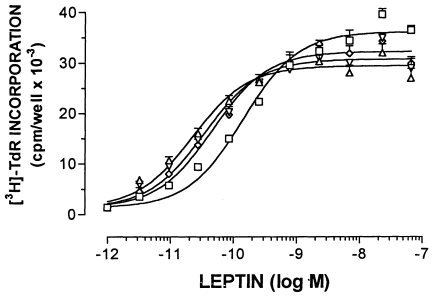

Fig. 5. Biological activity of ovine leptin-Q (△), ovine leptin-SF (▽), human leptin-Sf9 (□) and mouse leptin-His^6 (◇) in BAF/3 cells transfected with the long form of human leptin receptor. DNA synthesis was monitored by ^{3}H-TdR incorporation. The results represent mean ± S.D. ($n = 3$). For other details see ref. [12].

combinant ovine leptin which will allow future in vivo experiments.

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