Production and Refolding of Recombinant Leptin

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The product of the obese (ob/ob) gene, leptin, is a hormone produced by adipocytes and is a major regulator of appetite and metabolism. The ob/ob mutant mice produce a truncated leptin protein because of a mutation of the ob/ob gene (9). Consequently, these animals exhibit obesity, hyperinsulinemia and infertility (1). The mechanisms of action of leptin in regulating appetite, metabolism and fertility are under intense investigation and require the availability of leptin for such studies. Therefore, a convenient technique for the production of recombinant leptin is necessary. Most recombinant proteins are purified in a denatured state; therefore, refolding of the proteins and formation of correct disulfide bonds are required for obtaining their native bio logically active forms. We have attempted several refolding procedures that involve sequential dialysis with decreasing concentrations of urea (2.4), renaturation of the protein immobilized to affinity resin (6) and renaturation of the protein in a 20% glycerol buffer (5). These procedures led to the precipitation of the protein during refolding, producing very low yields. Although another protocol for mouse leptin purification and refolding has been described recently (7), we describe a more simplified protocol that requires only basic laboratory equipment for the purification and refolding of leptin that gives high purity, high yield and a biologically active product.

To obtain recombinant leptin, total RNA from mouse adipose tissue was isolated using TRI Reagent® (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer’s recommendations. The cDNA for the mouse leptin was amplified by reverse transcription polymerase chain reaction (RT-PCR). One microgram of total RNA was incubated with 50 ng of random hexamers at 70°C for 10 min and then at 4°C for 5 min. The RT reaction was carried out in a volume of 20 µL containing 1 µg total RNA, 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol (DTT), 0.5 mM each dNTP, 2.5 mg/mL of random hexamer primers, 20 U RNasin® (Promega, Madison, WI, USA), 200 U of SUPERSCRIPT™ II RNase H- Reverse Transcriptase (Life Technologies, Gaithersburg, MD, USA) and incubated at 23°C for 10 min and 42°C for 60 min. The enzyme was inactivated by heating the reaction mixture at 70°C for 10 min. Primers containing a BamHI site were designed to amplify mouse cDNA from amino acids 22–167 (GenBank® Accession No. u18812) (5'-GGATCCGTGCGCTATCGAGATATCA-3’ and 5’-GGATCCGAGCAGGTGAGGCTAAACT-3’). One microliter from the RT reaction was amplified in a 50-µL reaction mixture containing 100 ng of each primer, 20 mM Tris-HCl, pH 8.8, 10 mM KCl, 10 mM (NH₄)₂SO₄, 2 mM MgCl₂, 0.1% Triton X-100, 0.1 mg/mL bovine serum albumin (BSA), 200 mM each dNTP and 2.5 U of Pfu DNA polymerase (Stratagene, La Jolla, CA, USA). An initial denaturation step at 94°C for 3 min was followed by 35 cycles at 94°C for 1 min, 60°C for 1 min and 72°C for 1 min, with a final extension at 72°C for 10 min. Amplifications were carried out in an AmpliTaq® II Thermal Cycler (Barnstead/Thermolyne, Dubuque, IA, USA). The 450-bp amplified product was resolved in a 1.5% MetaPhor® agarose gel. The DNA was visualized by ethidium bromide fluorescence. The amplified region was first cloned into the pGEM®-T vector (Promega) and subsequently subcloned into a BamHI site of the pQE-30 expression vector (Qiagen, Chatsworth, CA, USA) to introduce a 6xHis tag at the amino terminus of the protein. The pQE-30 plasmid was expressed in the M15 strain of E. coli bacteria containing pREP4 (Qiagen) re-
pressor plasmid. The cells were grown in LB medium containing 100 µg/mL ampicillin and 25 µg/mL kanamycin at 37°C to an absorbance of 0.7–0.9 at 600 nm. The expression of recombinant leptin was induced with 2 mM isopropyl-β-D-thiogalactopyranoside (IPTG; Promega) for 5 h. The cells were harvested by centrifugation at 4000×g for 20 min, and the pellet obtained was stored at -20°C. The protein was purified by homogenization of the cell pellet under denaturing conditions in a buffer containing 6 M guanidine HCl, 0.1 M sodium phosphate, 0.01 M Tris-HCl at pH 8.0 and at 5 mL of buffer per gram. Leptin was purified by metal chelate affinity chromatography on a nickel-nitrilotriacetic acid (Ni-NTA) resin (Qiagen), which has high selective binding for proteins with 6×His tags. The protein homogenate was loaded onto 8 mL of a 50% resin slurry previously equilibrated in homogenization buffer, stirred at room temperature for 45 min and then loaded onto a 1.6-cm-diameter column. The resin column was washed as described above to avoid intermolecular disulfide bond formation. The protein was then treated with 10 mM reduced glutathione and 1 mM oxidized glutathione dissolved in 5 mL of the refolding buffer. The refolding process was carried out by stirring slowly at room temperature for 16 h. After refolding, the pH of the solution was brought to 7.4 with glacial acetic acid, and the solution was then concentrated by ultrafiltration using a 50-mL Amicon ultrafiltration cell and Diaflo membrane (MWCO 3000) to a final concentration of 1 mg/mL. The purified and refolded leptin was filtered through a 0.2-µm filter, and purity was checked by SDS polyacrylamide gel electrophoresis (PAGE) as indicated previously. Using this protocol, the estimated recovery after refolding was greater than 80%–90% (Figure 1). To establish that the purification and refolding procedure did not alter the biological activity of the recombinant leptin, the refolded protein was administered to ob/ob female mice (0.15 mg/kg/day by Alzet® 1007-D Minipumps [Alza, Palo Alto, CA, USA] implanted subcutaneously), and its ability to decrease body weight, a known function of leptin (8), was determined. After 13 days of treatment, animals administered the purified and refolded mouse leptin showed a 30% decrease in body weight as compared to PBS-treated controls (Figure 2), thus verifying that the protocol yields a purified, refolded, biologically active leptin protein. A recent protocol describing purification and refolding of recombinant human leptin addressed the importance of differences in biological potencies of recombinant leptin preparations produced in E. coli (3). We report a refolding protocol that produces a recombinant mouse leptin with biological potency comparable to that saline (PBS) buffer (4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄, 137 mM NaCl, 2.7 mM KCl, pH 7.4) at 4°C for 24 h with three buffer changes. The protein solution was finally concentrated once more by ultrafiltration using a 50-mL Amicon ultrafiltration cell and Diaflo membrane (MWCO 3000) to a final concentration of 1 mg/mL. The purified and refolded leptin was filtered through a 0.2-µm filter, and purity was checked by SDS polyacrylamide gel electrophoresis (PAGE) as indicated previously. Using this protocol, the estimated recovery after refolding was greater than 80%–90% (Figure 1). 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described by Fawzi et al. (3).

In conclusion, the protocol described in these studies provides a simple and efficient method for purifying and refolding of the protein hormone leptin. Key steps in the protocol appear to be the gradual lowering of the denaturing agent and keeping the concentration of the protein low during refolding. These steps help avoid precipitation of the protein, thus ensuring high yields of the purified, refolded, biologically active leptin. This protocol should prove to be extremely useful to scientists in the leptin field who need to obtain high yield of pure and biologically active recombinant leptin for use in biological testing.

REFERENCES


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Pedro L. Zamorano, Liesl De Sevilla, Virendra B. Mahesh and Darrell W. Brann
Medical College of Georgia
Augusta, GA, USA