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High production and optimization of the method for obtaining pure recombinant human prolactin

Regina Affonso*, Carlos Roberto Soares, Maria Teresa Ribela, Paolo Bartolini

Centro de Biotecnologia, Instituto de Pesquisas Energéticas e Nucleares, São Paulo, Brazil

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ABSTRACT

Prolactin is a pituitary hormone that is involved diverse physiological functions, such as lactation, reproduction, metabolism, osmoregulation, immunoregulation, and behavior. Its level of glycosylation is low *in vivo*, which favors its expression in bacterial systems. In the present work recombinant human prolactin (rec-hPRL) was expressed from the p1813-hPRL vector in *Escherichia coli* strain in inclusion bodies with 530.67 mg of rec-hPRL per liter of induced bacterial culture. The solubilization and renaturation of rec-hPRL followed by two methods described in the literature for this protein: one with detergent and basic pH, and other urea and dialyses was done by studying. The protocol with detergent/basic pH was not successful, whereas protocol with urea/dialyses was obtained pure protein and this was optimized. Rec-hPRL was obtained in a soluble, pure and active form, when the sample was 8-fold concentrated in the solubilization phase, allowing 33% recovery, 3-fold more that the original method. The pure protein was obtained with 38.37 i. u./mg activity, which is three times greater than that of the PRL standard from the WHO. In conclusion, this work obtained the highest production of rec-hPRL, and concentrating the sample eight times in the solubilization stage was decisive for obtaining a highly concentrated, active protein for future work.

1. Introduction

Prolactin (PRL) is a member of a family of related pituitary hormones, such as growth hormone (GH) and placental lactogen (PL), that have similarity in the amino acid sequence and structural and biological features [1,2]. In vertebrates, PRL secretion is restricted to the pituitary, with PRL acting as a classical circulating hormone. It has diverse physiological functions and is involved in lactation, reproduction, metabolism, osmoregulation, immunoregulation, and behavior, among others [3]. In humans, in particular, it is produced by multiple tissues, where it is regulated in a cell-specific manner and acts as a cytokine [2].

Prolactin is single-chain protein that is expressed with a signal peptide of 28 amino acids, and the cleavage of this signal peptide results in the mature 23 kDa protein, which consists of 199 amino acids and contains three disulfide bonds [4]. This molecule has a single N-glycosylation site, which is glycosylated in 5–30% of its native pituitary form, but glycosylation is not essential for function [5].

Because its major form is not glycosylated, this protein was expressed in bacterial systems, in both the periplasm and cytoplasm [6–9]. Expressing human prolactin (hPRL) in inclusion bodies (IBs) may be a good strategy for obtaining material for multiple analyzes because as much as 90% of the protein in IBs is the recombinant protein. IBs have significant characteristics such as resistance to proteolytic degradation, the existence of native-like secondary structures of the expressed protein and easy separation of cellular debris using simple procedures [10,11]. Obtaining bioactive protein requires processing that involves its isolation from cells, solubilization, refolding and purification.

The methodology described in the literature to solubilize and renaturate prolactin or GH in IBs used chaotropic agents or detergents, such as 8 M urea, 6 M guanidinium hydrochloride and N-Laurylsarkosine [7,8,12,13].

Detergents are used for cleaning and to solubilize the aggregates of proteins. Luck et al. [7] in the purification process of the recombinant hPRL in inclusion bodies washed these with sodium deoxycholate to remove the membrane fragments, and they solubilized by low concentration of N-Lauroylsarkosine.

Guanidine hydrochloride is approximately 1.5–2x more potent than urea as a chaotropic agent [14], and the use of urea to solubilize may more appropriate because IBs contain a mixture of correctly and incorrectly folded recombinant protein [11]. Paris et al. [8] obtained recombinant human prolactin in a soluble bioactive form with urea as solubilizing agent and the renaturation by dialysis and gel filtration chromatography. Our aims were to obtain from a vector/bacterial

* Corresponding author. Biotechnology Center, IPEN-CNEN, Av. Prof. Lineu Prestes, 2242, Cidade Universitária, 05508-000, São Paulo, Brazil.
E-mail address: reginaffonso@yahoo.com.br (R. Affonso).

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system that allows efficient expression of recombinant proteins and to establish protocols to obtain large amounts of pure proteins with biological activity.

2. Materials and methods

2.1. Expression conditions of p1813-hPRL vector

The transformed *E. coli* HB2151 containing the p1813-hPRL plasmid [15], were grown in LB medium (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl) supplemented with kanamycin (50 μg/mL) at 37 °C while shaking at 180 rpm. When the OD_{600} reached 0.4–0.8, the cells were induced with 0.1 mM isopropyl-β-D-thiogalactoside (IPTG, Sigma-Aldrich, Missouri, USA) and cultured for 9 h at 37 °C while shaking at 180 rpm. All of the cultures were centrifuged at 4000 g for 5 min at 4 °C, and the pellets were processed or stored at −20 °C [16]. The induced and non-induced cultures were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [17] and Western blotting [18].

2.2. Solubilization of inclusion bodies and renaturation of rec-hPRL

The pellets from the induced culture were suspended in buffer (50 mM Tris-HCl and 0.5 mM EDTA, pH 8), sonicated seven times for 30 s each on ice (60 kHz) and then centrifuged at 12,000 g for 15 min at 4 °C. The pellets were washed four times with TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8) and processed or stored at −20 °C.

Solubilization was performed by using either of three protocols:

a) 5 mg of rec-hPRL was suspended in 8 mL of buffer (0.1 M phosphate buffer, pH 7.4, containing 1 mM DTT and 0.1 mM PMSF); it was adjusted to 1% sodium deoxycholate and incubated for 1 h at 37 °C. Next, it was centrifuged and washed once with water. The pellet was resuspended in 0.1 M sodium phosphate buffer pH 7.4 containing 0.2% N-Lauroylsarkosine and incubated overnight at 4 °C. Then, the sample was clarified by centrifugation for 15 min at 12,000 *g* at 4 °C [7].
b) 10 mg of rec-hPRL was suspended in 100 mL of urea buffer (0.2 M phosphate buffer, pH 7, containing 8 M urea and 1% 2-β mercaptoethanol) at a ratio of 1:10, then the solution was heated at 55 °C for 5 min and incubated at room temperature for 2 h [8].
c) 10 mg of hPRL was suspended in 12.5 mL of the same urea buffer at a ratio of 1:1.25, optimized method. The protocol then followed the same procedure as before b.

Renaturation of the solubilized rec-hPRL were performed by basic pH and dialysis.

a) The sample containing 0.2% N-Lauroylsarkosine were diluted in buffer with 0.1 M sodium borate (pH 10.0) and 0.2% N-Lauroylsarkosine - 1:3, and incubated for 1 h at 20 °C. The sample renaturation occurred at pH 10.0 and in contact with the air. Then the solution was neutralized by addition of 6 N HCl, incubated for 1 h and centrifuged for 15 min at 12,000 g at 4 °C, and the supernatant was stored at 4 °C [7].
b) The samples containing urea were dialyzed against 100 volume of 50 mM NH_4HCO_3 at 4 °C for 36 h, and the buffer was changed eight times. At the end this time, the sample was centrifuged at 12,000 g for 15 min at 4 °C, and the supernatant was stored at 4 °C [8].

2.3. Protein purification

All renaturated rec-hPRL samples were purified using gel filtration chromatography; the protein eluted from a Sephacryl S-100 column (4.0 × 100 cm) (GE, São Paulo, Brazil) in 50 mM NH_4HCO_3 buffer at 4 °C at a flow rate of 60 mL/h and was monitored with a UV-1/124 monitor (GE, São Paulo, Brazil). Fractions containing rec-hPRL were pooled and analyzed by SDS-PAGE.

2.4. SDS-PAGE and western-blotting analyses

E. coli that were transformed with p1813-hPRL were analyzed by SDS-PAGE. These analyses were conducted using a 12% denaturing polyacrylamide gel and staining with Coomassie Blue (Sigma-Aldrich, Missouri, USA) [17].

For Western-blotting analysis, the proteins that separated in the polyacrylamide gel were transferred to a nitrocellulose membrane [18]. The membrane was incubated with polyclonal rabbit anti-hPRL antiserum (Santa Cruz Biotechnology, Dallas, USA) at a 1:500 dilution. The samples were then incubated with ^{125}I-labeled Protein A that had been prepared via the Chloramine-T method [19]. All samples were analyzed at a concentration of 4.8×10^7 cells/mL (OD_{600} = 0.06).

2.5. Protein quantification

The densities of the bands of the reduced pituitary hPRL standards and the densities of the corresponding bands of the rec-hPRL samples were determined using a computerized laser scanning densitometer (Model CS-9301 P C Dual Wavelength, Shimadzu, Japan). The amount of rec-hPRL in each extract, the renaturation and purification steps were then estimated by referencing a standard curve that had been constructed from the densities obtained with pituitary hPRL. Three trials were performed for all experiments (n = 3).

2.6. In vitro bioassay of lactogenic hormone

hPRL was assayed for lactogen activity in vitro by measuring the stimulation of the growth of lactogen-dependent rat Nb2 lymphoma cell cultures, following the procedure of Gout et al. [20]. Before their addition to the cultures, the rec-hPRL samples were diluted with Fischer's medium (Gibco, Grand Island, NY) containing 10% nonlactogenic horse (gelding) serum (National Biological Laboratory Ltd., Dugald, Manitoba, Canada). An International Standard of PRL (WHO, World Health Organization), with a bioactivity of 21.2 i. u./mg, was used as a lactogen standard [21]. Each sample was assayed at four to seven different concentrations that were selected to give a growth response within the useful working range of the assay, i.e., 0.0, 0.01, 0.03, 0.06, 0.12, 0.25 and 0.50 ng Standard PRL/mL [7].

2.7. Statistical analysis

Data were expressed as the mean ± S.E. of at least three independent experiments. Statistical significance was computed by using the unpaired Student's *t*-test. A $p \leq 0.05$ was conventionally considered statistically significant. (SE - standard error).

3. Results

3.1. Determination of expression efficiency of p1813-hPRL vector

The expression of rec-hPRL in the p1813-hPRL/HB2151 system was induced by different concentrations of IPTG (0.1, 1.5, 1.0 and 2.0 mM) and at seven activation times (3, 5, 6, 7, 8, 9 and 16 h) to determine the best rec-hPRL expression conditions (data not shown).

The production of rec-hPRL was high, resulting in 132.67 ± 0.33 μg/mL.A_{600} (530.67 mg/L), and the specific activity were also high of 0.52 ± 0.02, in the conditions: 0.1 mM IPTG and cultured for 9 h. Specific activity was calculated as percentage of rec-hPRL/total bacterial proteins.

Analyses of SDS-PAGE results confirmed the presence of a protein that was expressed from p1813-hPRL vector (Fig. 1 a) and this has the same molecular weight as expected for rec-hPRL, Fig. 1 b. Its