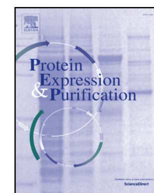
Protein Expression and Purification 152 (2018) 131–136

Contents lists available at ScienceDirect

Protein Expression and Purification

journal homepage: www.elsevier.com/locate/yprep



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

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ARTICLE INFO

Keywords:
Recombinant human prolactin
High production
Inclusion bodies
Optimization in solubilization
Biological activity

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

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https://doi.org/10.1016/j.pep.2018.07.015
Received 20 July 2018; Accepted 30 July 2018
Available online 01 August 2018
1046-5928/ Published by Elsevier Inc.

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

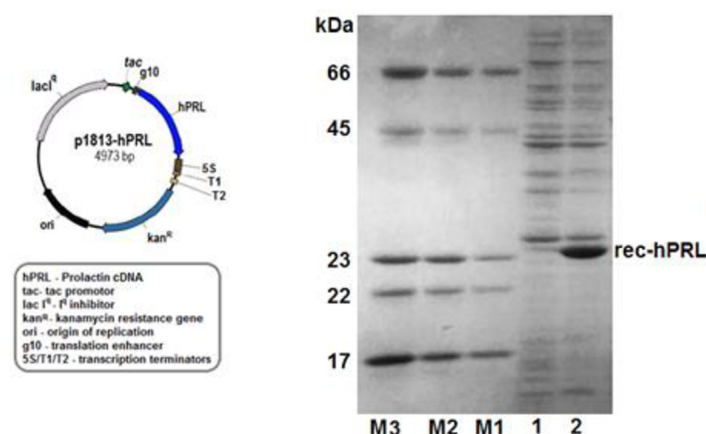

Fig. 1. a) Schematic representation of the p1813-hPRL vector showing the location with a description of its elements. b) and c) Analyses of rec-hPRL samples expressed in cultures with the p1813-hPRL vector induced for 9 h at 37 °C. Lanes M3, M2 and M1 contained 3, 2 and 1 μg of protein marker, respectively. The 23 kDa protein is pituitary PRL that was used as standard. **b)** SDS-PAGE: line 1, non-induced culture and line 2, induced culture. **c)** Western blotting: line M3, 23 kDA pituitary PRL standard; lines 1 and 2, induced and non-induced cultures.

immunologic activity is shown in Fig. 1 c, in which there is a hPRL dimmer above the prolactin in the hPRL standard (line M3) and induced culture (line 1). Western blotting analysis showed hPRL dimmers both in standard and in the recombinant sample, band above hPRL.

3.2. Analyses of solubilization of inclusion bodies and renaturation of rec-hPRL

The rec-hPRL expression of 132.67 μg/mL.A_{600} was used in all solubilization and renaturation experiments. The first protocol used was of Luck et al. [7], which used 1% sodium deoxycholate with incubation for 1 h at 37 °C and then solubilized with 0.2% N-Laurylsarkosine. The result obtained with 1% sodium deoxycholate was the solubization of all rec-hPRL, Fig. 2 a.

In Fig. 2 a, lines 6 and 7 are samples with deoxycholate buffer, in which the rec-hPRL was solubilizated and in this step the sample was discarded. The sample in line 8, in which should be the rec-hPRL, there is nothing, evidencing the solubilization by deoxycholate buffer not working. In this point, the deoxycholate buffer was removed from the protocol.

The methodology that used N-Lauroylsarkosine and basic pH allowed the production of apparently pure and soluble protein, Fig. 2 b. Although rec-hPRL was in soluble form, Fig. 2 b lines 4 and 5, when it was purified using gel filtration chromatography, it was eluted in the Vo, Vo corresponds to the molecular exclusion volume of the column, Fig. 2 b line 6 and Fig. 2 c.

Table 1 shows the concentration of rec-hPRL obtained in the process steps with the use of sodium deoxycholate and without this detergent.

Paris et al. [8], which uses urea as a solubilizer in samples with 1 mg of hPRL/10 mL of buffer, and the renaturation was performed via dialysis. The renaturation method produced 50.81 ± 0.91 μg/mL.A_{600}, and after purification by Sephacryl S-100 column the amount of pure rec-hPRL were 14.67 ± 0.52 μg/mL.A_{600} (Table 2).

In this study, the rec-hPRL:volume ratio of the urea buffer was reduced to 1:1.25, an eight-fold concentration. In this modified protocol, there was a loss of hPRL in the solubilization step, but this loss was compensated for in the following renaturation step. The amount of rec-hPRL protein that was obtained at the end of the purification was 43.88 ± 1.97 μg/mL.A_{600}, approximately three times more than was produced by the original method.

In the method of Paris et al. [8], Fig. 3 a, the recovered rec-hPRL is almost all aggregated, Vo; the monomer rec-hPRL appears at volume Ve at a concentration of 14.67 ± 0.52 μg/mL.A_{600} with a recovery of 11%.

The rec-hPRL was purified using the optimized method, and better recovery was achieved, as shown in Fig. 3 b. In this figure, the Vo fraction contains rec-hPRL at a concentration of 35.01 ± 0.31 μg/mL.A_{600}, and the Ve_1 fraction of 43.88 ± 1.97 μg/mL.A_{600} is purified

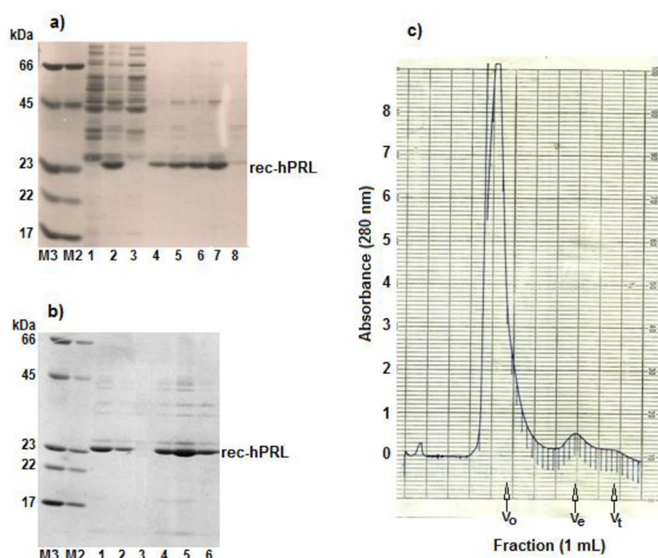

Fig. 2. SDS-PAGE analyses of rec-hPRL samples expressed in cultures with the p1813-hPRL vector induced for 9 h at 37 °C and the steps of solubilization and renaturation by Luck et al. [7] protocol. Lanes M3 and M2 contained 3.0 and 2.0 μg of protein marker, respectively; and the 23 kDa protein is pituitary PRL that was used as standard. **a)** line 1, non-induced culture; line 2, induced culture; line 3, bacterial lysate supernatant after centrifugation - discarded; lines 4 and 5, inclusion bodies suspension; lines 6 and 7, inclusion bodies suspension in deoxycholate buffer - discarded; line 8, inclusion bodies suspension in N-Lauroylsarkosine buffer. **b)** lines 1 and 2 inclusion bodies suspension; line 3, bacterial lysate supernatant after centrifugation - discarded; line 4 and 5, inclusion bodies suspension in N-lauroylsarkosine buffer; line 6, void volume (Vo) from Sephacryl S-100 column sample. **c)** elution patterns from Sephacryl S-100 column of rec-hPRL. The sample was eluted with 50 mM NH_4HCO_3 buffer and flow rates were 1 mL/fraction. The void volume (Vo) was 81.5 mL, the total volume (Vt) was 186.5 mL, and the eluted volume (Ve) was 166.5 mL.

protein with a recovery of 33%. SDS-PAGE analysis of Ve_2 showed that it did not contain protein (Fig. 3c, line 7).

In this Fig. 3 c shows all steps of optimized method, in which the rec-hPRL is pure after of the purification, line 6.

3.3. In vitro bioassay of lactogenic hormone

The rec-hPRL protein was evaluated for its biological lactogenic activity by using Nb2 cell culture assays (Fig. 4). The comparison of the activity of rec-hPRl with that of the PRL standard (from the WHO) shows that rec-hPRL has greater activity, 38.37 ± 0.42 i. u./mg, which is significantly higher than that of the PRL standard, 21.23 ± 0.24 i. u./mg ($p = 0.00012$).

Table 1
Rec-hPRL recuperation using the method from Luck et al. [7] with and without 1% sodium deoxycholate. IC = inclusion bodies.

Steps	With sodium deoxycholate		Without sodium deoxycholate	
	rec-hPRL (μg/mL.A_{600})	Recovery[a] (%)	rec-hPRL (μg/mL.A_{600})	Recovery[a] (%)
hPRL-IC	132.67 ± 0.33	100	132.67 ± 0.33	100
Deoxycholate	22.00 ± 0.52	17.58	–	–
Water	nd	–	119.40 ± 0.75	90
Solubilization	nd	–	96.85 ± 2.33	73
Renaturation	nd	–	53.07 ± 1.60	40

nd: not detected.
[a] Relative amount of initial rec-hPRL.

4. Discussion

The Laboratory of Pituitary Hormones has its own tradition in the studies of growth and prolactin hormones and the expression of their receptors in bacterial systems [6, 21–25]. In this study, rec-hPRL was expressed as inclusion bodies because they allow the easy isolation of product, produce a low level of bacterial contaminants, and produce high concentrations of protein that are free from the action of proteases [26,27].

The p1813 vector has been used for high expression in inclusion bodies, producing 500 mg/L of re-abg [28] and 376 mg/L of hRPL10 [15]. In this study, p1813-hPRL produced 530 mg of rec-hPRL of protein in inclusion bodies per liter of induced bacterial culture. These IBs were solubilized by two different processes which are describe of literature for prolactin: detergent and urea.

Ionic detergents such as SDS, N-Lauroylsarkosine and sodium deoxycholate are effective in solubilizing membrane proteins or IBs. Solubilization of proteins by detergents is dependent upon the formation of micelles in solution; which are stable above a critical concentration value or Critical Micellar Concentration (CMC). This value is specific to each detergent, in the case of the sodium deoxycholate this is 2.7 mM (20 °C) and for the N-Lauroylsarkosine is 13.7 mM (20 °C) (AppliChem – Detergents, 2008, www.applichem.com). In the results obtained with solubilization with detergents, washing with 1% of sodium deoxycholate that was to clean the sample, solubilized this one. This solubilization probably occurred why the CMC value was far above of 2.7 mM, this was 24 mM, which formed micelles that involved the IBs or aggregates making them soluble. Another increase for the action of sodium deoxycholate was the long incubation time and high temperature, for 1 h at 37 °C; we followed the protocol of Luck et al. [7] without changes. The methodology without deoxicholate was more successful in the recovery of rec-hPRL, Fig. 2 b.

Although the N-Lauroylsarkosine detergent has apparently solubilized the IBs, when this sample was analyzed by gel filtration chromatography, the sample was aggregated, Fig. 2 b (line 6) and 2 c. The concentration of N-Lauroylsarkosine used was 6.8 mM, below the limiting concentration for the formation of micelles that was 13.7 mM. One possibility for this result may be detergent inefficiency due to low buffer concentration, partially solubilizing rec-hPRL. The elution volume Ve = 166.5 mL, Fig. 2 c, is not the same for rec-hPRL Ve = 118.5 mL, Fig. 3 c, the fact that the molecular dynamics of the formation of micelles or detergent aggregates may have caused this result. The hydrophobic regions of the detergent monomers may not be buried or point to the center of the micelle, packing in a disorganized fashion, forming small aggregates [29].

Luck et al. [7,30] studied the function of disulfide bridges and renaturation condition, and these authors were not intended to evaluate the purity of the sample. The study, which involved site-directed mutation, studied methodologies for renaturation by Western blotting and activity assays. This work did not mention problems with sodium deoxycholate buffer; however, mutations and disulfide bridges may interfere with the solubilization of bovine prolactin.

The solubilization and renaturation of rec-hPRL from inclusion bodies by urea and dialysis was efficient, with recovery of ∼56 μg per liter of induced bacterial culture (11%). This methodology was similar to that used by Paris et al. [8], these authors obtained after of the purification two peaks: one corresponding to r-hPrl dimmers and the other with r-hPrl monomers. The recovery in the second peak of r-hPrl monomeric was 11.4 μg per mL of induced bacterial culture (18%), amount close to that found in the present work. However, when the volume was reduced eight times, the more concentrated rec-hPRL sample allowed for a higher recovery of pure protein from the size-exclusion chromatography (33%). The final sample of rec-hPRL 8-fold concentrated showed no increase in the formation of dimmers or aggregates. This may be due to the characteristics of the IBs themselves, such as containing a percentage of protein folded correctly [15], and the action of urea in the sample of 1 mg of rec-hPRL/10 mL of buffer, original protocol, the action of urea solubilizing the IBs was more efficient, unfolding the recombinant protein molecules. While in the concentrated sample, the action of urea may have been mild, preserving the correctly folded rec-hPRL existing in the IBs. This may justify obtaining 48% of monomeric rec-hPRL in the optimized sample, while in the non-concentration sample only 29% of monomeric rec-hPRL was obtained.

The pure rec-hPRL was compared with the International Standard of PRL from the WHO in all characterization assays. SDS-PAGE and Western blotting analyses of the rec-hPRL protein confirmed its molecular weight and immunogenicity. Signal transduction pathways that use PRL depend on its activity, and this activity depends on its tertiary conformation as given by three disulfide bridges. Doneen et al. [31]

Table 2
Rec-hPRL recuperation using the method from Paris et al. [8] and this study (optimized method). IC = inclusion bodies.

Methods Steps	Paris et al. [8]		This study	
	rec-hPRL (μg/mL.A_{600})	Recovery[a] (%)	rec-hPRL (μg/mL.A_{600})	Recovery[a] (%)
hPRL -IC	132.67 ± 0.33	100	132.67 ± 0.33	100
Solubilization	112.77 ± 1.39	85	98.40 ± 1.93	74
Renaturation	50.81 ± 0.91	38	90.70 ± 1.11	68
Eluted	14.67 ± 0.52	11	43.88 ± 1.97	33

[a] Relative amount of initial rec-hPRL.

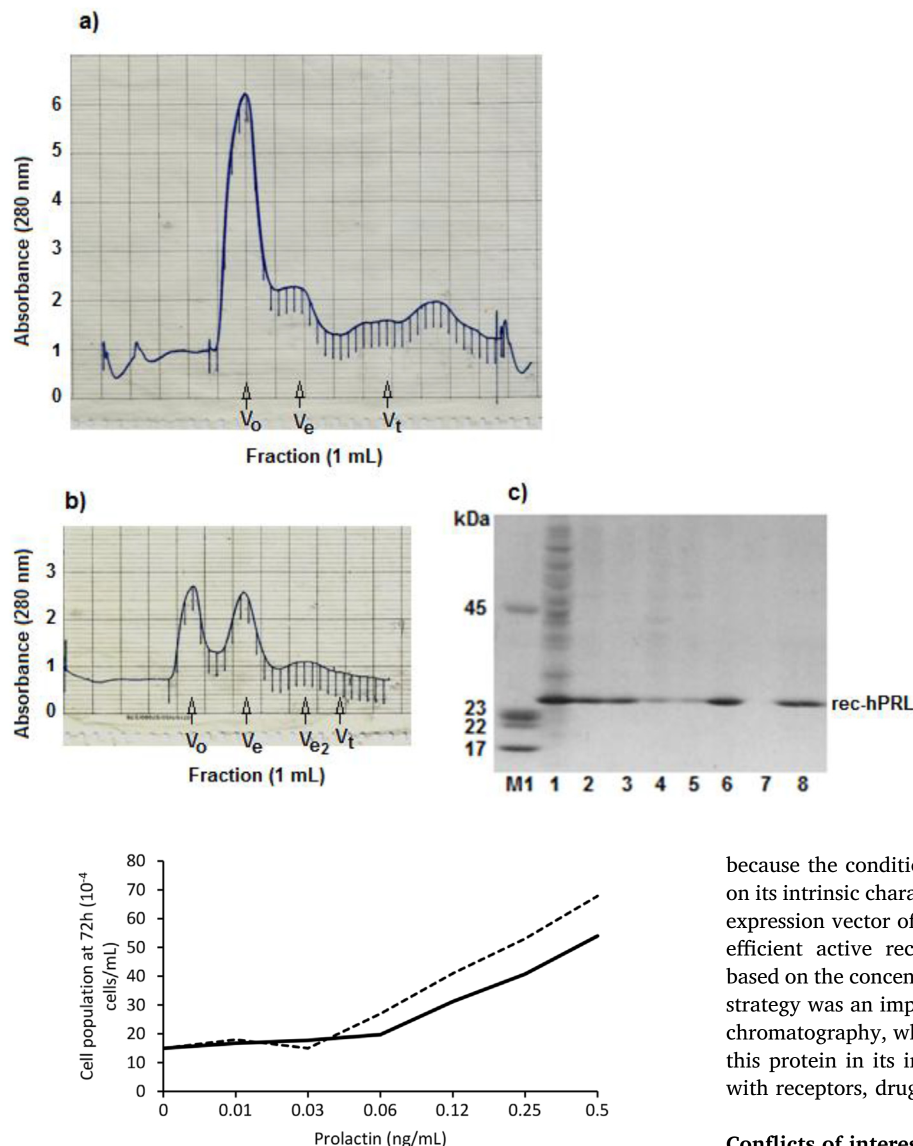

Fig. 3. Elution patterns from Sephacryl S-100 column of rec-hPRL. The column was equilibrated, and the sample was eluted with 50 mM NH_4HCO_3 buffer; flow rates were 1 mL/fraction. The void volume (Vo) was 81 mL, the total volume (Vt) was 186 mL, and the eluted volume (Ve) was 118.5 mL **a)** Paris et al. [8] method and **b)** optimized method. In **c)** SDS-PAGE analyses of rec-hPRL samples in the steps of solubilization, renaturation and purification by optimized protocol. Lane M1 contained 1,0 μg of protein marker: line 1, induced culture; line 2, inclusion bodies suspension; lines 3, solubilized and dialyzed rec-hPRL; line 4, pellet discarded from centrifugation product after dialysis; line 5, void volume sample; line 6, Ve_1 sample; line 7, Ve_2 sample; line 8, sample with urea buffer.

Fig. 4. Biological activity of rec-hPRL. The biological activity of rec-hPRL was compared with that of the International Standard of PRL by determining their stimulation of rat Nb2 lymphoma cell growth. Cultures (1×10^5 cells) were incubated for 72 h with doses of hPRL standard (S-hPRL) or recombinant hPRL (rec-hPRL). Solid line is WHO-PRL; dashed line is rec-hPRL. (n = 3).

studied the action of these disulfide bridges and observed in mutants ovine prolactin (oPRL) that loss of activity occurred when there was no formation of the three disulfide bridges; however, the presence of one or two of the disulfide bridges resulted in normal biological activity in oPRL. In the Nb2 assay shown that it is possible that the structural conformation of rec-hPRL is correct, with three intramolecular disulfide bonds between Cys 4–11, Cys 58–174 and Cys 191–199; the biological activity was significantly higher than of the Standard ($p = 0.00012$). We believe that this activity is due to the protein's high degree of purity and the absence of PRL isoforms, which are present in the International Standard (these are obtained from pituitary extracts and contain glycosylated and truncated forms).

5. Conclusion

Reviews describing recombinant protein production systems help in the design of a system that is suitable for expressing proteins of interest. However, choosing the perfect combination is not feasible a priori because the conditions under which each protein is expressed depend on its intrinsic characteristics. In the present work, p1813 vector is high expression vector of recombinant protein, and here was established an efficient active rec-hPRL recovery protocol that improvement was based on the concentration of the sample in the solubilization step. This strategy was an important point in purification by molecular exclusion chromatography, which generally elutes very dilute samples. Obtaining this protein in its intact, active form is critical for studies of binding with receptors, drugs or in hormonal dosing tests.

Conflicts of interest

The authors have no conflicts of interest.

Acknowledgements

We thank the members of the Centro de Biotecnologia by experimental support; and the National Commission of Nuclear Energy (CNEN) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for financial support.

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