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Expression, purification and characterization of the authentic form of human growth hormone receptor antagonist G120R-hGH obtained in *Escherichia coli* periplasmic space

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ABSTRACT

The human growth hormone receptor antagonist G120R-hGH precludes dimerization of GH and prolactin receptors and consequently JAK/STAT signaling. Some modifications in this antagonist resulted in a drug specific for the GH receptor, called Pegvisomant (Somavert®). However, the original G120R-hGH is usually synthesized in bacterial cytoplasm as inclusion bodies, not being a commercial product. The present work describes the synthesis and characterization of G120R-hGH secreted into bacterial periplasm and obtained with a vector based on a constitutive lambda-PL promoter. This antagonist can be useful for studies aiming at investigating the effects of a simultaneous inhibition of GH and prolactin signaling, as a potential anti-tumoral or anti-diabetic compound. G120R-hGH, synthesized using the W3110 *E. coli* strain, showed a yield of 1.34 ± 0.24 μg/ml/A_{600} (~0.79 mg G120R-hGH/g of wet weight cells) after cultivation at 30 °C up to 3 A_{600} units and induction at 37 °C, for 6 h, with final 4.3 ± 0.3 A_{600}. A laboratory scale purification was carried out using three chromatographic steps with a total yield of 32%, reaching 98% purity. The obtained protein was characterized by SDS-PAGE, Western Blotting, Mass spectrometry, RP-HPLC, HPSEC and *in vitro* proliferation bioassay. The proliferation assay, based on Ba/F3-LLP cells, shows that G120R-hGH (100 ng/ml) significantly inhibited (64%) the proliferative action of hGH (1 ng/ml). This is the first time that G120R-hGH is synthesized in bacterial periplasmic space and therefore correctly folded, without the initial methionine. The reasons for a divergent efficacy for antagonizing hGH versus hPRL is currently unknown and deserves further investigation.

1. Introduction

Human growth hormone (hGH) is a protein synthesized by the pituitary gland with 191 amino acids (aa), two disulfide bonds and a molecular weight of 22 kDa [1]. It belongs to the family of cytokines, binding to the hGH receptor and resulting in a complex formed by 1 molecule of ligand and 2 molecules of the receptor. Human GH can also bind to the human prolactin (hPRL) receptor. The discovery of an antagonist of hGH receptor resulted in a drug called Pegvisomant (Somavert®), already approved for treatment of acromegaly [2,3].

The main mutation is the substitution of the amino acid glycine for arginine at position 120 (G120R-hGH) responsible for its antagonistic effect. This amino acid modification changed the conformation of the molecule, allowing its binding to Site-1 of the hGH receptor and preventing, due to steric hindrance, its binding to Site-2 [3,4]. This molecule was later conjugated to a 5 kDa polyethylene glycol (PEG) in order to increase its circulatory half-life from 15 min to 100 h and, because PEG binds to the primary amino group of lysine residues, the original G120 was replaced by lysine (K), with similar antagonist effects [3]. In addition, eight amino acid were changed to increase the affinity for the hGH receptor and decrease the affinity for the hPRL receptor [3,5].

Since the GH/IGF-1 axis has been related to the progression of several types of cancer, an antagonist of hGH was studied as an

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anti-cancer therapeutic agent [3]. Additionally, scientific evidence has shown that an excess of prolactin increases the risk of developing obesity and type 2 diabetes mellitus (T2DM), while suppression of prolactin secretion has a positive impact on glucose homeostasis and energy balance [6]. Notably, several prolactin receptor antagonists have been synthesized by specific amino acid replacements in the protein chain of prolactin [7]. Nevertheless, these antagonists have never been studied in the context of the treatment of T2DM and obesity. Because hGH also has lactogenic effects similar to those of hPRL, sharing the same signaling pathway, G120R-hGH acting as a lactogenic antagonist may have interesting and useful therapeutic applications.

G120R-hGH was first synthesized in mouse L cells [8]. Thereafter, it was produced in CHO cells adapted to suspension culture in serum-free culture [9]. The cytoplasmic expression in *E. coli* (strain Rosetta) inclusion bodies, using IPTG for activation and urea for refolding, was reported by Langenheim et al. (2006) [10].

We describe here the first synthesis of G120R-hGH in bacterial periplasmic space, opening the way for future applications as a potential anti-tumoral or anti-diabetic compound. The synthesis in the periplasmic space has the advantage of producing a protein without the initial methionine, cleaved together with the signal peptide when the protein is transported from the cytoplasm to the periplasm, thus obtained in its authentic and correctly folded form.

2. Materials and methods

2.1. Construction of the plasmid

G120R-hGH synthesis in the periplasm of *E. coli* basically follows the same procedures already used for hGH synthesis [11]. The original signal peptide from hGH, with 26 amino acids, was replaced by the sequence of DsbA (a bacterial disulfide oxidoreductase) signal peptide with 19 amino acids and the codon "GGC" corresponding to amino acid 120, glycine, was replaced by "CGC", corresponding to arginine. With these modifications, the theoretical value of molecular mass for the G120R-hGH (191 aa) and of his precursor DsbA-G120-hGH (210 aa) are 22228.1 Da and 24200.7 Da, respectively. The cDNA containing the restriction sites of *Nde*I and *Bam*HI enzymes, DsbA signal peptide and G120R-hGH gene was synthesized by GenScript Corporation (Piscataway, NJ, USA) in the pUC57 vector. The plasmid λPL–DsbA–G120R-hGH was obtained by replacing the gene of mouse prolactin with the G120R-hGH gene in the plasmid λPL–DsbA–mPRL [12], as shown in Fig. 1. After having confirmed the correct sequence of the G120R-hGH gene, *E. coli* W3110 and RRI strains were transformed via the calcium

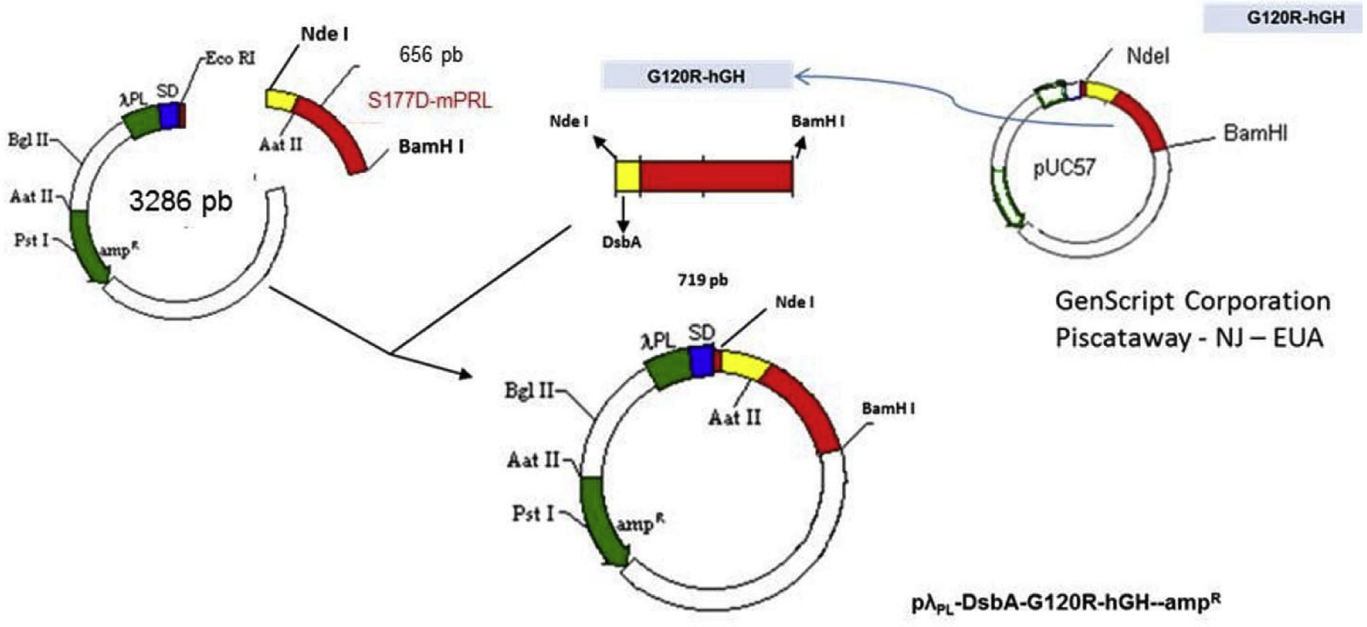

Fig. 1. Construction scheme of the G120R-hGH expression vector, pλPL-DsbA-G120R-hGH-amp^R (3223 bp) from pλPL-DsbA-S177D-mPRL-amp^R. λPL, major leftward promoter of bacteriophage λ; SD, Shine Dalgarno sequence.

Table 1
Influence of the temperature on G120R-hGH secretion into the periplasmic space. A comparison between RRI and W3110 strains of *E. coli* is shown. Cultivation up to a cell density of 3.0 A_{600} units at 30 °C was followed by a 6 h expression phase at different temperatures.

Induction Temperature (°C)	W3110 Strain G120R-hGH (μg/ml/A_{600}) mean ± sd	RRI Strain G120R-hGH (μg/ml/A_{600}) mean ± sd
30	1.02 ± 0.14 (n = 3)	0.41 ± 0.09 (n = 4)
32	1.02 ± 0.26 (n = 4)	0.81 ± 0.07 (n = 4)
35	1.04 ± 0.38 (n = 3)	0.91 ± 0.15 (n = 4)
37	1.60 ± 0.26 (n = 4)	0.95 ± 0.10 (n = 3)
42	1.40 ± 0.06 (n = 3)	0.53 ± 0.24 (n = 3)

chloride method [13] and selected for ampicillin resistance.

2.2. Expression at different temperatures

According to previous studies from our research group, the major leftward promoter of the bacteriophage λ (λP_L) was used as a constitutive promoter, since the plasmid responsible for the synthesis of a thermolabile repressor (pRK248clts) was not present [14,15]. This strategy has shown excellent results, particularly in the expression of temperature-sensitive proteins [14,15], but even for proteins like hGH that are considered to be thermally stable and that present good expressions at 42 °C. The absence of the repressor provided better yields and permitted the choice of a more efficient expression temperature (manuscript in preparation). We therefore decided to employ this same methodology in the present work. A transformed clone of each strain (W3110 or RRI) was cultivated in 250 ml Erlenmeyer flasks with 100 ml of Luria-Bertani medium (LB) with 100 μg/ml ampicillin. Incubation started at 30 °C with rotational shaking (150 rpm). After obtaining a biomass equal or superior to 3 A_{600} units, the synthesis of G120R-hGH was induced by raising the temperature for 6 h. We tested different temperatures: 32 °C, 35 °C, 37 °C and 42 °C. Quantification of G120R-hGH was performed in the periplasmic fluid by reversed phase high performance liquid chromatography (RP-HPLC) as described [16].

2.3. Osmotic shock

Periplasmic osmotic shock fluid was obtained via a modification of the method of Koshland and Botstein [17], as previously described [11]. Briefly, the product from fermentation broth is harvested and the bacteria are precipitated by centrifugation at 3000 *g* for 5 min. All subsequent steps were carried out at 4 °C in an ice bath. Pellets were resuspended in ice-cold 10 mM Tris-HCl, pH 7.5 adding 1 ml of 20% sucrose (w/v) for each 100 A_{600} units. Then 33 μl 0.5 M EDTA, pH 8.0, were added for each ml of solution and incubation on ice continued for 10 min. The suspension was centrifuged again at 3000 g for 10 min, the pellet being resuspended by adding 1 ml of ice-cold 1 mM Tris-HCl, pH 7.5 per each 100 A_{600} units, incubating 10 min, and centrifuging again at 3000 g for 10 min. The collected supernatant represents the periplasmic fluid.

2.4. G120R-hGH purification

The periplasmic extract obtained by osmotic shock was purified using ion exchange and size exclusion liquid chromatography [18]. As a first purification step, a strong anion exchange resin (Q Sepharose Fast Flow in a 16 × 125 mm column) was utilized, under a linear gradient of 0–1 M NaCl in 0.01 M ammonium acetate buffer, pH 7.0, with a flow rate of 2 ml/min, 10 column volumes (10 × 25 ml). The second purification step was a size exclusion chromatography (S100 Sephacryl resin, 26 × 100 mm column), run under isocratic conditions and eluting with 0.05 M ammonium bicarbonate buffer, pH 7.9, with a flow rate of 60 ml/h. For both steps, an ÄKTA system (GE Health Sciences, Buckinghamshire, UK) was used. In a third step, another size exclusion chromatography was performed on a HPLC system using a stainless steel TSKgel G2000SW column (60 cm × 7.5 mm ID, with a particle size of 10 μm and pore size of 125 Å) (TosoHaas, Montgomeryville, PA, USA), coupled with a TSKgel G2000SW guard column (7.5 cm × 7.5 mm ID, particle size of 10 μm). As mobile phase, 0.025 M ammonium bicarbonate, pH 7.0, was used at a flow rate of 1 ml/min, at room temperature, monitoring at a wavelength of 220 nm.

2.5. Protein determination

Total protein concentration was estimated using the Micro BCA protein assay (Pierce Chemical Co, Rockford - IL, USA), and a standard curve ranging from 0.5 to 200 μg/ml, constructed with a BSA

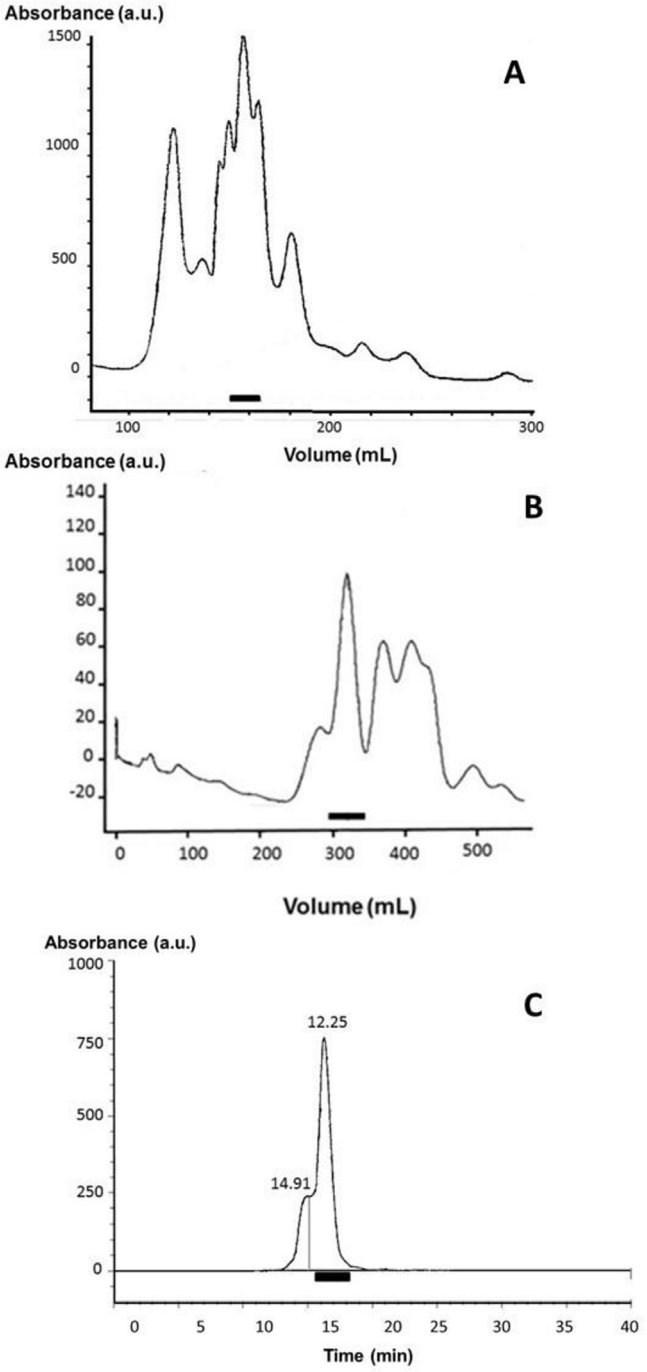

Fig. 2. G120R-hGH purification. **A**. First step, the periplasmic extract obtained by osmotic shock was chromatographed on a Q-Sepharose fast flow anion-exchange column (11 cm × 1.6 cm ID) equilibrated in 0.01 M ammonium acetate buffer, pH 7.0. G120R-hGH was eluted with a 0–1 M sodium chloride gradient, 10 column volumes (10 × 25 ml), at a flow rate of 2 ml/min, collecting fractions of 5 ml/tube. **B**. Second purification step, on a size-exclusion column (Sephacryl S-100). **C**. Third purification step, by HPSEC, the number above each peak indicates the correspondent retention time in minutes. The black bars correspond to G120R-hGH elution: fractions #14, #15 and #16 in A, fractions #37 to #45 in B and #16 to #18 in C.

solution (Sigma, Saint Louis - MO, USA).

2.6. Gel electrophoresis and Western blotting

Discontinuous SDS-PAGE, based on 12% polyacrylamide gels, was carried out as described by Laemmli [19] under non-reducing conditions. Coomassie Brilliant Blue G-250 (USB, Cleveland, OH, USA) was used for staining and the molecular mass markers were from GE Healthcare BioSciences (Uppsala, Sweden). For the Western blotting, the technique of semi-dry transfer on a nitrocellulose membrane was used, the analysis being performed by chemiluminescence (Luminata™ Forte Western HRP Substrate, Merck-Millipore, Darmstadt, DA, Germany) and exposure to X-ray film (Amersham™ Hyperfilm™ ECL, Fisher Scientific, Pittsburgh, PA, USA). A rabbit anti-hGH primary antibody (1:1000), an anti-rabbit IgG secondary antibody produced in goat and horseradish peroxidase (HRP) conjugate, (1:10000) were used (Signalway Antibody LLC, College Park, MA, USA).

2.7. Reversed–phase high-performance liquid chromatography

A Shimadzu Model SCL-10A high-performance liquid chromatography (HPLC) system coupled to a SPD-10AV UV detector (Shimadzu, Columbia, MD, USA), with Shimadzu class VP software and

Table 2
Recovery of G120R-hGH from *E. coli* W3110 periplasmic fluid after each purification step.

	Total protein[a] (mg)	G120R-hGH[b] (mg)	Mass fraction (%)	Recovery (%)
Periplasmic fluid	815	17.4	2.13	–
Q Sepharose FF	51	11.4	22.4	66
Sephacryl S100	8.0	7.9	98.8	69
HPSEC	5.7[c]	5.6[c]	98.2[c]	71

[a] Determined by BCA.
[b] Determined by RP-HPLC.
[c] Determined by HPSEC.

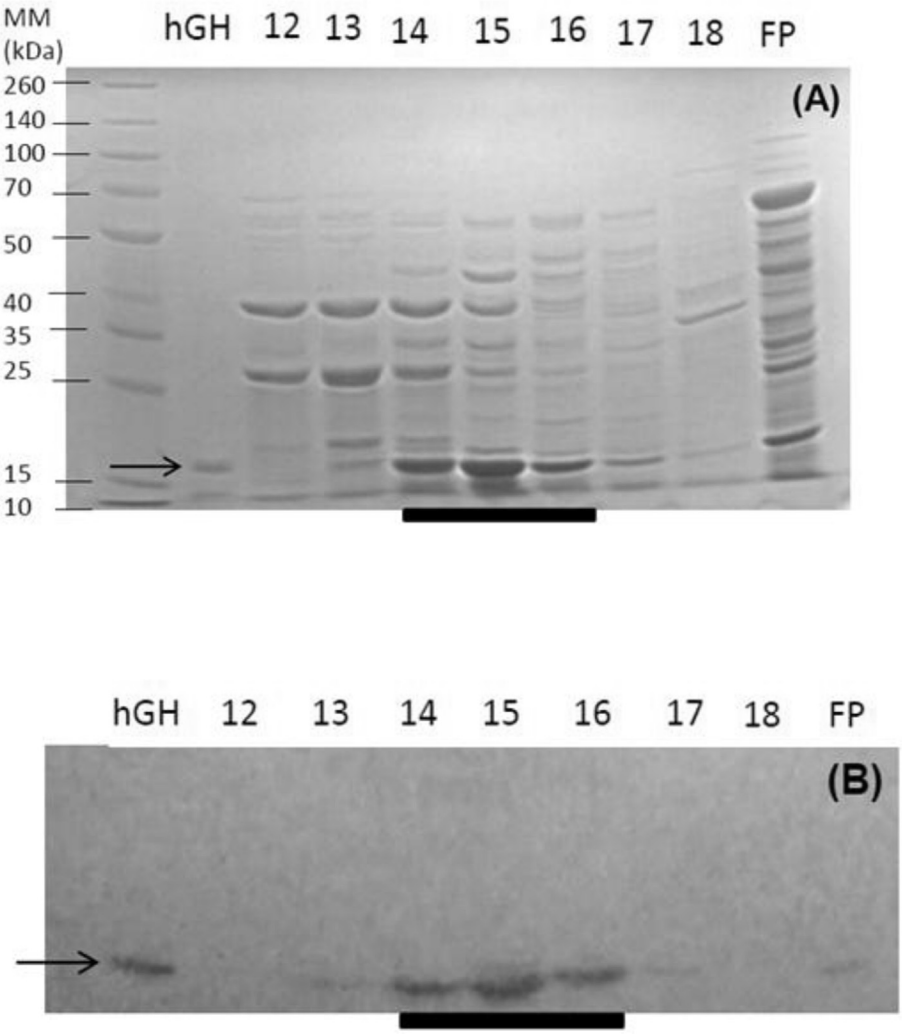

Fig. 3. A. SDS-PAGE analysis and **B**. Western Blotting, of fractions from the first purification step of G120R-hGH on the Q-Sepharose fast flow column. The arrows indicate the recombinant hGH (WHO 98/574) standard and the bars indicate fractions #14, #15 and #16, in which most of the G120R-hGH was present. FP, periplasmic fluid.

a Grace-Vydac C4 column 214TP54 (25 cm × 4.6 mm ID, pore diameter of 300 Å and particle diameter of 5 μm) (Hesperia, CA, USA) was used. To protect the main column, a silica column packed with 7.9–12.4 μm LiChrosorb Si-60 (Merck, Darmstadt, Germany) was installed. The mobile phase consisted of 71% Tris–HCl (50 mM, pH 7.5) and 29% n-propanol [16]. The flow rate was 0.5 ml/min, monitoring at a wavelength of 220 nm. The column temperature was maintained at 45 °C, and the maximum applied volume was 500 μL [15]. G120R-hGH was quantified against the International Standard of rec-hGH, coded WHO 98/574.

2.8. High-performance size-exclusion chromatography

A stainless steel TSK G2000SW column (60 cm × 7.5 mm ID, particle size of 10 μm, pore size of 125 Å) (TosoHaas, Montgomeryville, PA, USA) coupled to a TSKgel G2000SW guard column (7.5 cm × 7.5 mm ID, particle size of 10 μm) was used for analytical purposes with a mobile phase consisting of 0.025 M ammonium bicarbonate, pH 7.0, adjusted with 2% phosphoric acid. The flow rate was 1 ml/min, at room temperature, monitoring at a wavelength of 220 nm [15,16].

2.9. Electrospray ionization mass spectrometry (ESI-MS)

For molecular mass determination, a triple quadrupole linear hybrid ion trap spectrometer (MS/MS) (3200 QTrap, AB Sciex) was used with an Agilent 1260 HPLC (quaternary pump, degasser) and UV-VIS detector (1290) equipped with a Jupiter C4 chromatography

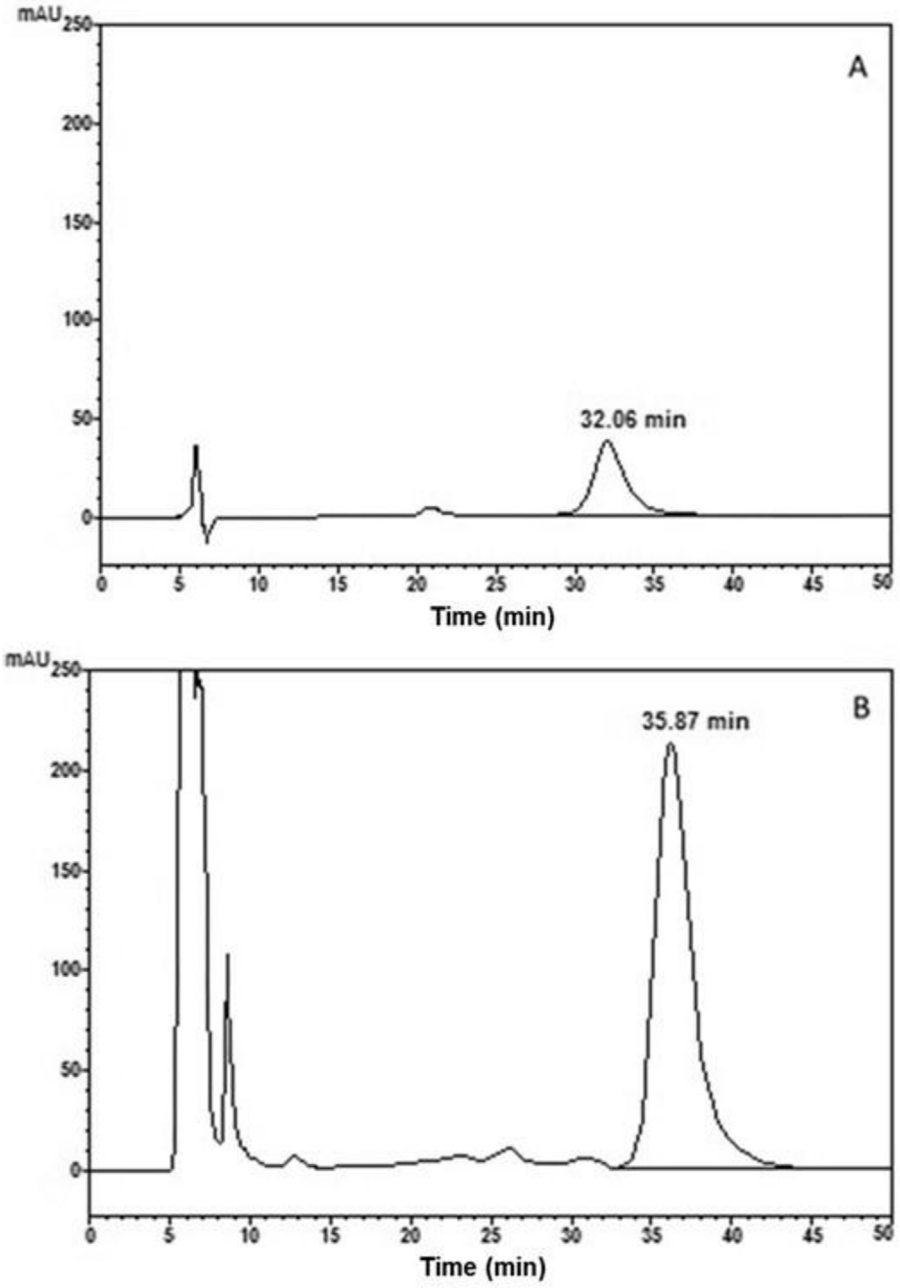

Fig. 4. Isocratic RP-HPLC (C4 Vydac 214 TP 54) analysis. **A**. Recombinant hGH (WHO98/574) standard, (t_R = 32.06 min); **B**. fraction #15 eluted from the Q-Sepharose fast flow column containing G120R-hGH, (t_R = 35.87 min).

column (250 mm × 4.6 mm ID, particle size of 5 μm, pore size of 300 Å) (Phenomenex, Torrance, CA, USA) at a flow rate of 0.6 ml/min, at 60 °C. Mobile phase A consisted of 95% water, 5% acetonitrile and 0.1% TFA, and mobile phase B of 95% acetonitrile, 5% water and 0.1% TFA. The mode of acquisition was positive electrospray - MS.

2.10. Biological assay

The G120R-hGH antagonistic activity was studied by a proliferation assay using mouse pro-B cells Ba/F3-LLP, as previously described [7,20]. Ba/F3-LLP cells transfected with the long hPRL receptor gene, were routinely maintained suspended in RPMI 1640 medium (Thermo Fisher Scientific, Waltham, MA, USA), supplemented with 10% fetal bovine serum (FBS) inactivated by heating (20 min at 45 °C), 2 mM glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin (Thermo Fisher Scientific, Waltham, MA, USA), 700 μg/ml Geneticin (G418, Sigma-Aldrich, St. Louis, MO, USA) and 1 ng/ml of a preparation of recombinant hPRL. Before starting the proliferation assay, cells were maintained in medium without prolactin and 1% inactivated FCS for 4–6 h, then distributed in 96-well plates (5×10^4 cells/well). The final volume, after sample addition, was 200 μl per well. After 72 h incubation at 37 °C and 5% CO_2, the number of viable cells was assessed by staining with MTS [21]. Briefly, 2 mg/ml MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolina (Promega Corp., Madison, WI, USA)] dissolved in phosphate buffered saline (PBS) were mixed at a ratio of 20:1 (v:v) with 0.92 mg/ml phenazine methosulfate (Sigma-Aldrich, St. Louis, MO, USA) in PBS. Twenty microliters of the mixture were added to each well and, after 2 h incubation at 37 °C, the absorbance was read at a wavelength of 490 nm, using a microplate reader (Multiskan EX, Thermo Electron Corp., Finland). The samples were analyzed in triplicate and in three independent assays.

3. Results and discussion

After confirming the correct construction of the plasmid λPL-DsbA-G120R-hGH by restriction analysis, it was used to transform W3110 and RRI *E. coli* strains, so the promoter λP_L was used as constitutive promoter.

Some limitations on the use of constitutive expression system based on a strong promoter from the phage lambda are, for example, increased segregational and structural instability and growth inhibition. Therefore, some authors defend that part of these problems can be easily circumvented and, in many cases, efficiently with the use of constitutive systems [12,14,22–24]. According to these authors, in fact, a constitutive system might be applicable under certain circumstances.

Menart et al. [22] have not observed any structural instability, due to delection of the repressor cI857 gene. There was, however, a significant impact on segregation stability in the absence of selection pressure. Menart et al. report that if the ampicillin level is maintained during fermentation, there is no significant difference between thermoinducible and constitutive systems. A selection pressure (100 μg/ml ampicillin) resulted in no significant changes in segregational stability. After ~200 generations, 90% of cells still harbored plasmids, practically the same when starting culture, being temperature-independent (30 °C or 42 °C).

Soares et al. (2008) reported a study carried out in shake flask cultures (LB medium, 30 °C), to verify plasmid losses. They determined human prolactin periplasmic yield in a strain lacking the repressor, after two growth periods corresponding to 10 and 50 *E. coli* generations. The specific expression was practically the same after the two growth periods. In addition, the presence or absence of antibiotic did not influence the specific expression yield for at least 40 generation.

In this work, to circumvent or minimize these problems, the *E. coli* clone was cultivated during the growth phase at lower temperatures (30 °C), in presence of ampicillin (100 μg/ml). In this condition, the rate of plasmid loss is possibly reduced due to the smaller copy number and existence of a selection pressure [22,25,26]. As mentioned, in the classical temperature-inducible system, the lambda P_L promoter is inhibited by the presence of the thermo-sensible repressor and only derepressed or switched on by increasing the temperature to 42 °C. In our system, in spite of the absence of the repressor, the promotor "induction", increasing G120R-hGH expression, also occurs as consequence of simply increasing temperature. This can be due also to other mechanisms that should be better investigated, such as the increase in the plasmid copy number, better metabolic efficiency of the bacteria protein synthesis, or by a more efficient action of the lambda PL promoter, whose activity, in a certain range, has been reported as inversely dependent by the temperature [27]. The increase in temperature also contributes to degradation of the protein of interest or formation of aggregates, resulting in a lower production. In fact, the level of accumulated G120R-hGH can be the result of an equilibrium between rate of protein synthesis and rate of degradation [14,28].

In the present work, we made a comparison between the two modified strains evaluating the expression at different temperatures, from 30 °C to 42 °C. RP-HPLC was used for the qualitative and

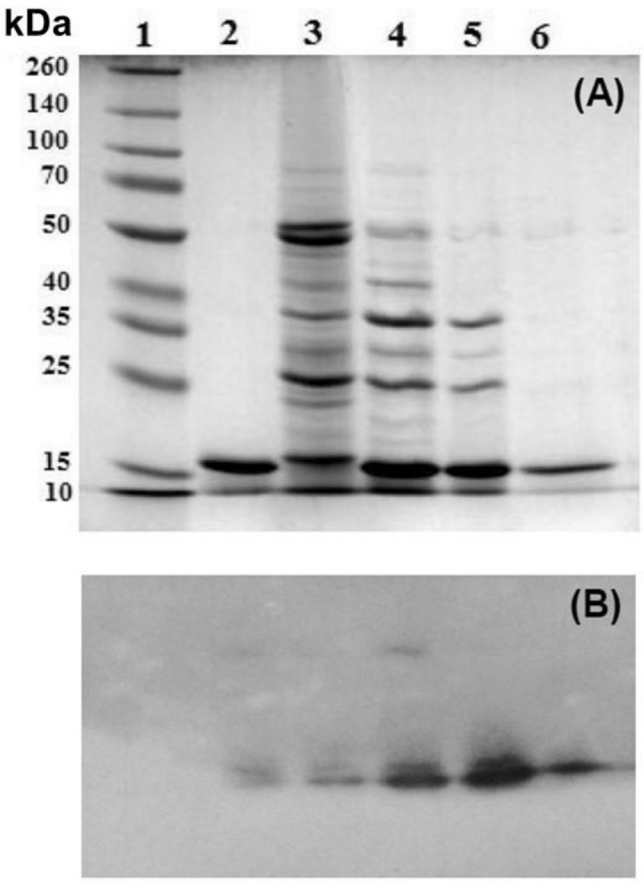

Fig. 5. Comparative analysis of all of the G120R-hGH purification steps. **(A)** SDS-PAGE under non-reducing conditions on a 12% polyacrylamide gel, stained with Coomassie Blue G. **(B)** Western blotting analysis of the same samples (1) molecular mass marker, (2) recombinant hGH (WHO 98/574 standard), (3) periplasmic fluid, (4) Q Sepharose FF pool, (5) Sephacryl S100 pool, (6) HPSEC pool.

quantitative analysis of the antagonist (Table 1). The results show that the highest expression (1.60 ± 0.26 μg/ml/A_{600}) was obtained in W3110, at 37 °C. It is interesting to note that, at 42 °C, the temperature used when the repressor pRK248cIts was present, periplasmic expression decreased. In this case, the absence of the repressor and consequent induction at 37 °C probably eliminated the side effects due to thermoinduction, such as the increased expression of heat shock proteins (e.g., proteases), activation of the

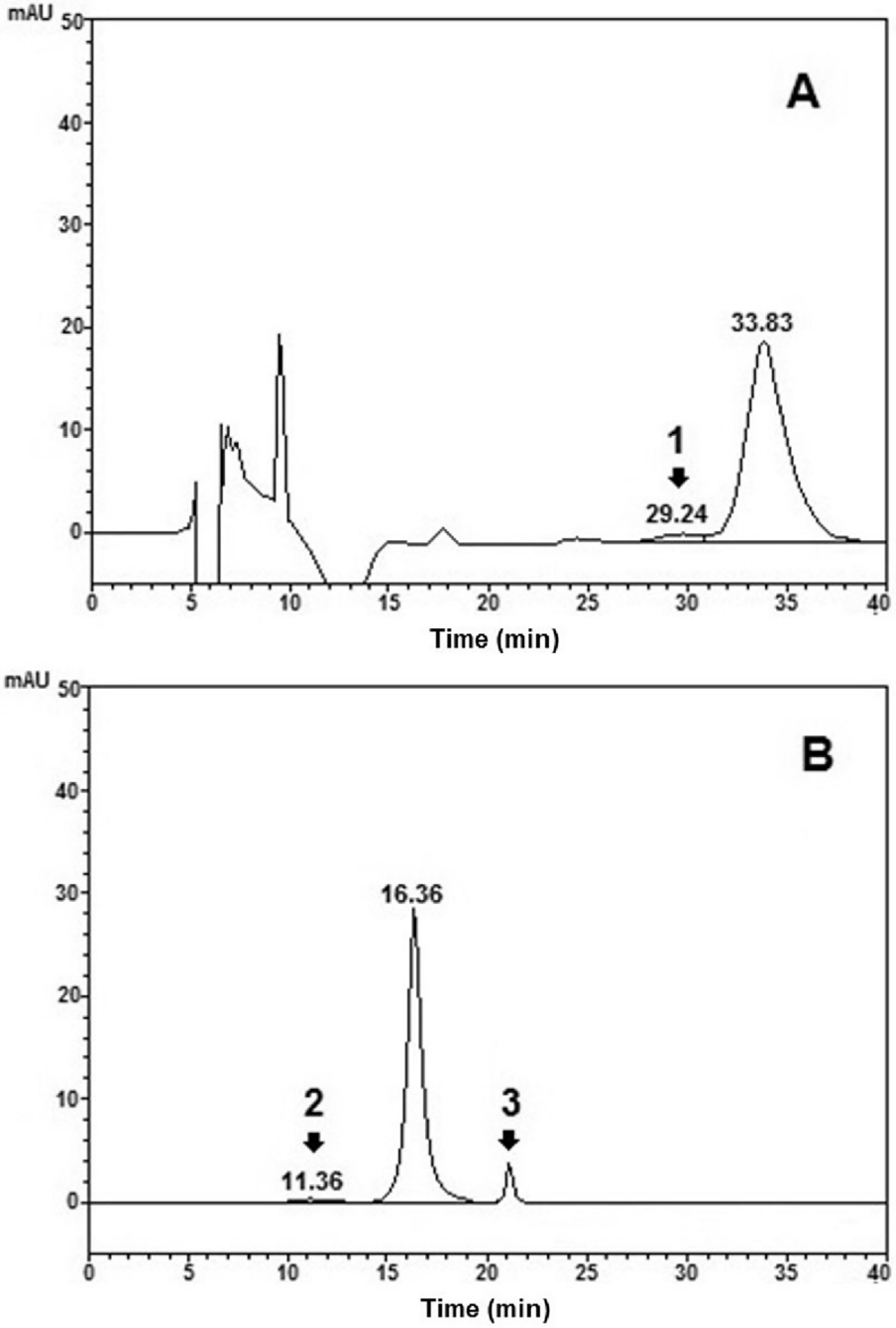

Fig. 6. Purified G120R-hGH analysis. **A**. RP-HPLC (t_R = 33.83 min). **B**. HPSEC (t_R = 16.37 min). The arrows indicate oxidized or deamidated forms (1), dimers or aggregated forms (2) and ammonium bicarbonate (3).

Table 3
Molecular mass determination by electrospray MS/MS of hGH (WHO 98/574) international standard, of hPRL internal standard and of G120R-hGH.

Protein	Theoretical value of molecular mass (Da)	Experimental value of molecular mass (Da)	Error (%)
hGH	22129.0	22129.0	0.0
hPRL	22898.1	22901.5	+0.015
G120R-hGH	22228.1	22240.0	+0.053

SOS response, significant cell lysis, etc [22,23]. Other protein syntheses, based on the same system, have been reported [14,15]. Constitutive expression systems are less used in comparison with inducible expression of heterologous proteins but, in the case of nontoxic proteins, this system can be used, with the advantage of permitting fermentation at lower temperatures [14,15,22]. We chose the λPL-DsbA-G120R-hGH-W3110 clone, cultivated at 30 °C and then induced at 37 °C, to prepare 3 L of fermentation broth in Erlenmeyer flasks, in three lots of 1 L. The final optical density was 4.3 ± 0.3 A_{600}, with a yield of 1.34 ± 0.24 μg/ml/A_{600} or of approximately 0.79 mg G120R-hGH/g of wet weight cells, considering that an optical density of 1 A_{600} results in 1.7 g wet weight cell/L [29].

A three step purification process was developed to obtain pure G120R-hGH from the periplasmic fluid. The first purification step was an ion exchange chromatography. It was necessary to concentrate the product approximately 16-fold for a partial purification (Fig. 2A; Table 2). SDS-PAGE, Western Blotting (Fig. 3A and B) and RP-HPLC analysis (Fig. 4) permitted identification of the fractions with the greatest amount of G120R-hGH, which constituted the appropriate pool from this first purification step. This pool was used for the second purification step, a size exclusion chromatography on Sephacryl S-100 (Fig. 2B). SDS-PAGE analysis was used again to choose a pool containing G120R-hGH for the next purification step, preparative HPSEC (Fig. 2C). Each purification step had a recovery >65% and the final yield of the whole purification process was 32.2% (Table 2). Fig. 5A shows SDS-PAGE analyses for each purification step and the progressive reduction of the high molecular weight contaminants. The immunological activity of the product was confirmed by using rabbit anti-hGH antibody (Fig. 5B). The final product showed 3% oxidized and/or deamidated forms, as determined by RP-HPLC (Fig. 6A), and a purity of ~98% by HPSEC

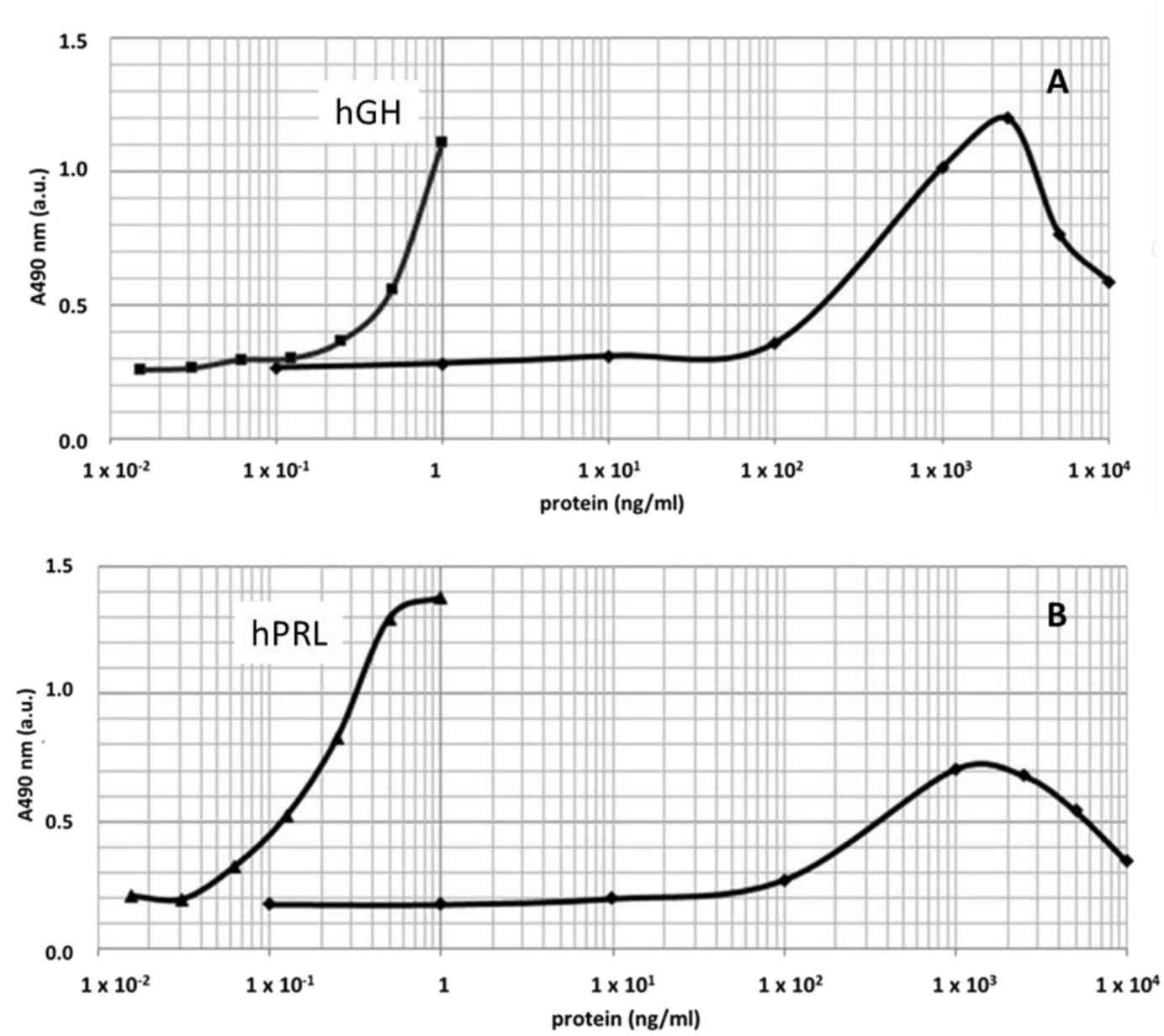

Fig. 7. Typical dose response curve based on the Ba/F3-LLP cell proliferation bioassay. (A) Agonistic response of G120R-hGH in comparison with hGH (WHO 98/574) international standard; (B) Agonistic response in comparison with hPRL internal standard. -■- hGH (ng/ml); -▲- hPRL (ng/ml) and -◆- G120R-hGH (ng/ml).

(Fig. 6B).

Electrospray ionization mass spectrometry (ESI-MS) provided the expected mass of G120R-hGH, with a value of 22240 Da, which is only 0.05% higher than the theoretical value of 22228.1 Da (Table 3). This result confirms the exceptional accuracy and precision offered by mass spectrometry, as already mentioned in previous work [12]. This analysis is important, considering that the mass difference of 0.4% (99.1 Da) existing between G120R-hGH and hGH cannot be detected by SDS-PAGE, WB or HPSEC techniques. A difference in hydrophobicity between the antagonist and authentic hGH of approximately 12%, was however detected by RP-HPLC (Fig. 4), providing an interesting identity parameter for this hGH variant. A similar difference (~10%) in hydrophobicity was reported for hPRL in relation to its antagonist G129R-hPRL [7]. It is interesting to note that the greater hydrophobicity observed for the antagonist is not directly due to the amino acid substitution in itself, considering the shift from a glycine (nonpolar) to arginine, a positive charged amino acid. As reported by Soares et al. (2006) [7], for hPRL and its analog antagonist G129R-hPRL, this hydrophobicity increase seems to be a consequence of secondary and tertiary structure alterations.

For the *in vitro* biological activity determination of G120R-hGH, a proliferative assay was employed because an increase in cell division is among the easiest biological responses to quantify. The reference bioassay for lactogens is the Nb2 cell proliferation assay [30]. However, the use of rat cells raises the problem of species specificity when analyzing human proteins, as in our case. A proliferative assay, obtained by stably transfecting pro-B Ba/F3 cells with a plasmid encoding the long isoform of hPRL receptor, was used. This homologous lactogenic assay (Ba/F3-LLP) uses the so-called Low Low Prolactin (LLP) cell population [7,20]. Its sensitivity is similar to that of the Nb2 assay and is about ten times higher than the original Ba/F3-LP assay [31].

G120R-hGH showed a weak agonist activity, as shown in Fig. 7 and reported by others authors [31,32]. While the maximum proliferation activity achieved for hPRL corresponded to 0.3 ng/ml and 1 ng/ml for hGH, the maximum G120R-hGH proliferative effects occurred with ~3 μg/ml, i.e., with a 3000-fold higher concentration. Some studies using G120K-hGH with Ba/F3 LP cells did not observe this effect, probably because of the lower sensibility of the bioassay used [31]. We observed a pronounced bell-shaped dose-response curve for the antagonist, reflecting self-antagonism at higher concentrations (>3 μg/ml), a phenomenon reported in detail by Bernichtein et al. [31].

Regarding the antagonism effects on hPRL-R, the presence of 100 ng/ml G120R-hGH significantly inhibited (64%) the proliferative action of 1 ng/ml hGH (Fig. 8A). Nevertheless, only a small inhibition (7%) was observed in the case of hPRL (Fig. 8B). Although the reason for this is unclear, it might be due to a stronger binding of hPRL to the long receptor isoform in comparison with hGH binding, which could be influenced by the presence of zinc [2,31–34]. In order to observe a higher *in vitro* antagonism versus hPRL it could be necessary to increase greatly the concentration of G120R-hGH.The bell-shaped effect might, however, hide the response. In fact, bioassays with high sensitivity to low concentrations of lactogens might not be appropriate for evaluating the antagonistic properties of mutated ligands [31], as in the case of Ba/F3-LLP cells.

In work recently published by our research group, the distribution of prolactin, mGH and hGH-responsive neurons was characterized in the mouse brain. Our findings revealed an extensive amount of prolactin and GH responsive neurons and a significant interaction between these hormones in several brain structures related to the regulation of metabolism and glucose homeostasis [35]. The synthesis of G120R-hGH in *Escherichia coli* periplasmic space described here may provide a valuable tool for investigating the effects of this antagonism in several types of cancer, as well as in the regulation of energy balance and glucose homeostasis.

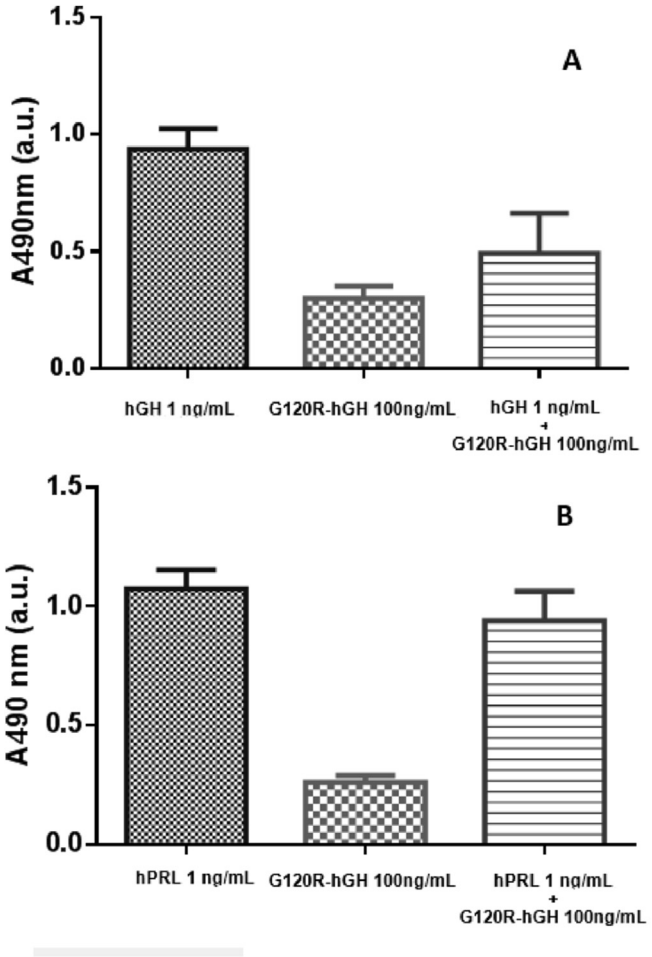

Fig. 8. Antagonist effect of G120R-hGH in the proliferation assay based on BaF/3-LLP cells. (A) Antagonist effect on hGH; (B) Antagonist effect on hPRL.

4. Conclusions

In conclusion, the expression of the hGH receptor antagonist G120R-hGH in the periplasmic space is reported for the first time. Purification from periplasmic fluid provided a 98% pure product and a quantity adequate for characterization after three chromatographic steps. The laboratory production process developed here yielded 5 mg of G120R-hGH from 3 L of culture medium. The product was characterized by physico-chemical and immunological methods and its proliferative and antagonistic activity were evaluated *in vitro* against hGH and hPRL. G120R-hGH will be used for future biological studies in animal models related to obesity and diabetes.

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