

# Distinct human prolactin (hPRL) and growth hormone (hGH) behavior under bacteriophage lambda P<sub>L</sub> promoter control: Temperature plays a major role in protein yields

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## Abstract

When producing recombinant protein for therapy, it is desirable not only to obtain substantial amounts of the protein, but also to make sure that potential contaminants such as inducing agents are not present in the final product. To prevent this, one can use expression systems in which the promoter ( $\lambda P_L$ ) is activated by a temperature shift that denatures a repressor (e.g., cIts). In this manner, hGH was successfully expressed and secreted in *Escherichia coli* periplasm, with specific yields well above  $1 \mu\text{g ml}^{-1} A_{600}^{-1}$ , after a temperature shift from 30 to 42 °C. However, attempts to express a related hormone, human prolactin, employing the same protocol were unsuccessful, providing  $0.03 \mu\text{g ml}^{-1} A_{600}^{-1}$  at the most. A process is described in which this labile protein is obtained from a cIts<sup>-</sup> strain under optimized temperature condition (37 °C). The highest periplasmic secretions of prolactin ever reported were thus obtained:  $0.92 \pm 0.10 \mu\text{g ml}^{-1} A_{600}^{-1}$  at an optical density of  $\sim 3 A_{600}$  units in shake flask cultures and  $\sim 1 \mu\text{g ml}^{-1} A_{600}^{-1}$ , at an OD of 35  $A_{600}$  units, via a rapid and flexible batch feed process in laboratory bioreactor. Purified hPRL was monomeric, correctly processed (Mr = 22,906), properly folded and bioactive ( $51.5 \pm 24.1 \text{ IU mg}^{-1}$ ).

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**Keywords:** Human growth hormone; Human prolactin; *Escherichia coli* periplasm; Lambda P<sub>L</sub> promoter; Thermo-sensitive repressor; Fed batch culture

## 1. Introduction

Prolactin (PRL), a protein hormone primarily produced by the anterior pituitary, has various physiological functions in mammals. It is best known for its stimulation of lactation and its regulatory roles in the growth and differentiation of the mammary gland and in reproduction (Clarke and Bern, 1980; Sinha, 1995; Goffin et al., 2002, 2005).

While human PRL (hPRL) presents different isoforms as a result of pre- or post-translational modifications (e.g. glycosy-

lation), the basic hormone is a single-chain polypeptide with a calculated molecular mass of 22,897.75 Da, which has been confirmed by relative molecular mass (Mr) determinations (Wu et al., 2003; Soares et al., 2006). It consists of 199 amino acids and three disulfide-bonded loops and is the primary form in human circulation (Sinha, 1995).

Since abnormal levels of hPRL have been linked to a number of disorders in the areas of pituitary function and reproduction (e.g., hyperprolactinemia, galactorrhea, infertility), it is one of the hormones most frequently determined in routine clinical assays (Kirby et al., 1979; Morganti et al., 1996; Glezer et al., 2006). Consequently, there is a considerable need for highly purified hPRL in the preparation of *in vitro* diagnostic reagents. Although routine therapeutic applications of either hPRL or its analogs/antagonists (Soares et al., 2006) have not yet been established, former studies indicate several possible applications of this hormone (Soares et al., 2000), while more recent studies in rats have shown that PRL levels increase in response to stress, helping to protect against acute stress-induced hypocalcemia and gastric erosions (Fujikawa et al., 2004). Zhang et al. (2005), report that recombinant hPRL (rhPRL) promotes antitumor

**Abbreviations:**  $A_{600}$ , units of absorbance at a wavelength of 600 nm; CHO, Chinese hamster ovary; cIts, thermo-sensitive repressor of the P<sub>L</sub> promoter of bacteriophage  $\lambda$ ; CRS, Chemical Reference Standard; DsbA, a 21 kDa *E. coli* periplasmic protein; IPTG, isopropyl-beta-D-thiogalactopyranoside; hGH, human growth hormone; hPRL, human prolactin; rhPRL, recombinant human prolactin;  $\lambda P_L$ , major leftward promoter of the bacteriophage  $\lambda$ ; LB, Luria–Bertani medium; Mr, relative molecular mass; PAGE, polyacrylamide-gel electrophoresis; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate;  $t_A$ , activation temperature (in °C);  $t_R$ , retention time

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effects of human NK cells transferred to tumor-bearing SCID mice. At present, different clinical trials on rhPRL applied to lactation induction are underway ([www.clinicaltrials.gov](http://www.clinicaltrials.gov)). In view of the above, an increasing need for pure, biologically active, authentic hPRL can be anticipated.

Several laboratories have reported expression of hPRL in different prokaryotic or eukaryotic host cells and the strategies that have been applied for the synthesis of this and other human pituitary hormones are described in a recent review (Ribela et al., 2003). An interesting strategy for obtaining authentic hPRL is bacterial periplasmic expression, a cheaper alternative compared to mammalian cells expression (Price et al., 1995) that, as far as we are aware, has only been described by our group (Morganti et al., 1998; Ueda et al., 2001; Soares et al., 2002). Periplasmic specific expression yields higher than  $0.1 \mu\text{g ml}^{-1}$   $A_{600}^{-1}$  were, however, never reported. Periplasmic secretion of recombinant proteins offers a multitude of advantages when compared to other expression systems in *Escherichia coli* and in mammalian cells (e.g., CHO). Among these, we can mention: low contaminants, low proteolytic activity and lower amounts or even absence of undesirable isoforms such as high molecular weight, glycosylated, phosphorylated or cleaved forms. Comparing directly with cytoplasmic expression, the periplasm contains only 4% of the total cell proteins, which makes purification considerably less onerous. The cleavage *in vivo* of the signal peptide provides, moreover, the authentic N terminus of the target protein (i.e. without an extra methionine) while the oxidizing environment of the periplasm facilitates the proper folding of the protein (Makrides, 1996; Soares et al., 2003). Despite all the desirable features of this secretion system, some heterologous proteins fail to be successfully expressed in the periplasm, limiting this technique to certain selected candidate products (Soares et al., 2006).

In our laboratory, high periplasmic expression levels of human growth hormone (hGH) had been achieved by using a system based on the control of  $\lambda P_L$  promoter by the thermo-sensitive cIts regulator (Soares et al., 2003). Indeed, this is one of the most widely used promoters for *E. coli* large-scale protein production. This promoter was chosen based on cost and health/safety concerns since thermal induction is free of chemical inductors such as IPTG or nalidixic acid, which are expensive and potentially hazardous for personnel and the environment and thus less attractive for large-scale production of human therapeutic proteins (Makrides, 1996; Soares et al., 2003). This thermo-inducible promoter is activated at temperatures around  $42^\circ\text{C}$  and fully repressed at  $30^\circ\text{C}$ . The major shortcomings of this temperature shift methodology are the problems commonly associated with protein expression above  $37^\circ\text{C}$  (e.g., proteolysis, degradation, aggregation) that can negatively influence the production of particularly labile proteins.

Considering the improved hGH expression presented by the system utilizing the signal peptide of DsbA, we decided to use it also for authentic hPRL expression and secretion (Soares et al., 2003). After preliminary poor results, in view of the fact that hPRL has been considered a particularly labile protein (Nyberg et al., 1985; Parlow, 1997; Morganti et al., 1998; Soares et al., 2002) we planned the synthesis of periplasmic hPRL by utilizing

the vector containing the  $\lambda P_L$  promoter, but without the presence of the repressor protein, and under optimized temperature conditions.

## 2. Material and methods

### 2.1. Plasmid constructions

The DNA sequence, including the restriction endonuclease site Nde I, start codon, DsbA signal peptide, hPRL cDNA coding for the native sequence of the hormone, stop codon and Bam HI site, was obtained by polymerase chain reaction (PCR) utilizing the plasmid p3SN8hPRL3 (Morganti et al., 1998) as a template. The synthetic primers used for this PCR were: forward primer, including the Nde I site, DsbA signal peptide and 20 nt of the hPRL cDNA sequence (5'-GAT-CGA-TCG-ATC-CAT-ATG-AAA-AAG-ATT-TGG-CTG-GCT-CTG-GCT-GGT-TTA-GTT-TTA-GCG-TTT-AGC-GCA-TCG-GCG-TTG-CCC-ATC-TGT-CCC-GGC-GG-3'); reverse primer, including a C-terminal sequence of the hPRL gene and the Bam HI site, downstream of the stop codon (5'-GAT-CGA-TCG-GAT-CCT-TAT-CAG-CAG-TTG-TTG-TTG-TTG-AT-3'). After digestion of the DNA fragments obtained by PCR with the endonucleases Nde I and Bam HI, the fragments were subcloned into the Nde I–Bam HI sites of a cassette expression vector, the same utilized to obtain the hGH vector ( $\lambda P_L$ –DsbA–hGH) containing the  $\lambda P_L$  promoter and ampicillin resistance gene (Soares et al., 2003). The resulting hPRL vector was named  $\lambda P_L$ –DsbA–hPRL.

### 2.2. Bacterial strains and shake flask cultivation

*E. coli* RRI strain, carrying the low copy-number plasmid pRK248cIts (Bernard and Helinski, 1979) encoding for the thermo-sensitive transcription repressor (cIts) gene, was used for propagation and extraction of pRK248cIts. For hPRL or hGH expression, the *E. coli* host strain W3110, obtained from American Type Culture Collection (ATCC), was used. Four transformed *E. coli* W3110 strains were used: two harboring only the expression vector ( $\lambda P_L$ –DsbA–hPRL or  $\lambda P_L$ –DsbA–hGH), selected on LB agar plates supplemented with  $100 \mu\text{g}$  of amp  $\text{ml}^{-1}$ , and two strains harboring the expression vector plus the repressor gene-bearing plasmid pRK248cIts, selected on LB plates supplemented with  $100 \mu\text{g}$  of amp and  $50 \mu\text{g}$  of tetracycline  $\text{ml}^{-1}$ . Cultivation was carried out in 500 ml shake flasks containing 100 ml of Luria–Bertani (LB) medium. Each flask was inoculated with a single colony of *E. coli* cells taken from LB agar plates and incubated on a rotary shaker at  $30^\circ\text{C}$ . After growth to about  $1.5 A_{600}$ , the culture was either maintained at  $30^\circ\text{C}$  or heat-induced at different temperatures as described.

### 2.3. Batch and fed batch cultivation of the transformed W3110 *E. coli* strain

All batch and fed batch bioreactor experiments were conducted in a 201 Laboratory Bioreactor (New MBR, Zurich,

Switzerland) with the addition of  $100 \mu\text{g ml}^{-1}$  ampicillin. The bioreactor was operated at pH 7.2 and, to avoid  $\text{O}_2$  limitation, dissolved  $\text{O}_2$  was maintained above 20% of air saturation by increasing the agitation speed from 200 to 900 rpm and adding air at 1 VVM. A complex culture medium, which was a two- or four-fold concentrate of the HKSII medium described by Jensen and Carlsen (1990), was used. For inoculation, 500 ml (10% of the total initial volume) of LB medium that had reached  $0.5 A_{600}$  units were used, the culture being maintained at  $30^\circ\text{C}$  until activation. In the case of batch culture, after the absorbance reached about  $3 A_{600}$  units, the hPRL production was induced by raising the temperature to  $37^\circ\text{C}$  for 6 h. For fed batch, the conditions were similar to batch cultivation. As the carbon source, a glucose or glycerol feed in complex medium was started at a biomass concentration corresponding to  $5 A_{600}$  units. The feed rate was adjusted to the desired value and maintained constant until the end of the cultivation. Activation at either  $t_A = 37^\circ\text{C}$  or  $42^\circ\text{C}$  was started when  $A_{600}$  was reaching a plateau. The glucose feeding rate, when not specified differently, was  $3.6 \text{ g l}^{-1} \text{ h}^{-1}$  during the fed batch and  $5.4 \text{ g l}^{-1} \text{ h}^{-1}$  during the activation step.

#### 2.4. Osmotic shock

Periplasmic osmotic shock fluid was obtained via a modification of the method of Koshland and Botstein (1980), as previously described (Soares et al., 2003).

#### 2.5. Analytical size-exclusion (HPSEC) and reversed-phase (RP-HPLC) high-performance liquid chromatography

A Shimadzu Model SCL-10A HPLC apparatus coupled to a SPD-10AV UV detector (Shimadzu, MD, USA) was used, employing the Class VP software, also from Shimadzu. Columns and chromatographic methodologies were utilized as previously described (Dalmora et al., 1997; Soares et al., 2002, 2006). The WHO Chemical Reference Standard (CRS) of hPRL and the first WHO Reference Reagent for human PRL, recombinant, glycosylated (G-hPRL) 98/580 were used in the HPSEC purity test.

#### 2.6. HPSEC–radioimmunoassay (RIA)

For the determination of the percent of monomeric form of hPRL that is present in the periplasmic extracts, samples of osmotic shock fluids were analyzed via HPSEC and the collected fraction tested by an in-house hPRL-RIA, set up as previously described (Morganti et al., 1996).

#### 2.7. Western blot analysis

SDS-PAGE, based on 15% polyacrylamide gels, was carried out under non-reducing conditions and Western blot analysis was performed as described (Soares et al., 2006). The anti-hPRL antiserum (rabbit), NIDDK-anti-hPRL-3 (AFP-C11580), was provided by Dr. A.F. Parlow from the National Hormone and Pituitary Program (Torrance, CA, USA).

#### 2.8. Plasmid stability studies

To confirm stable maintenance of the plasmid vectors, the W3110 strain harboring the  $\lambda\text{P}_L$ -DsbA-hPRL was cultivated on LB broth (with and without ampicillin) for at least 50 generations, keeping it always in log phase at  $30^\circ\text{C}$ . hPRL expression and antibiotic resistance were compared to an analogous culture which was grown for only 10 generations.

#### 2.9. Prolactin purification

Purified prolactin (>97%), utilized for HPSEC purity test, for the *in vitro* bioassay and for ESI-MS, was obtained as previously described (Ueda et al., 2001), with purification yields of the order of 67–79%.

#### 2.10. *In vitro* bioassay

The bioactivity of purified hPRL was determined using the Nb2-lymphoma-cell-proliferation assay as described in previous work (Morganti et al., 1996) against the Chemical Reference Standard of recombinant hPRL (a  $2.2 \text{ mg ml}^{-1}$  solution, stored at  $-80^\circ\text{C}$ ) and the First WHO Reference Reagent for PRL, human, recombinant, lyophilized in ampoules coded 97/714 with a specific activity of  $57.2 \pm 11.4 \text{ IU mg}^{-1}$  (Rafferty et al., 2001).

#### 2.11. Electrospray ionization mass spectrometry (ESI-MS)

Previously dried  $5 \mu\text{l}$  ( $\sim 1 \mu\text{g}$  protein) samples were dissolved in 0.1% formic acid and applied to the automatic injector of a HPLC–ESI-MS system (MSQ Surveyor, Thermo Fisher Scientific, Waltham, MA, USA), under a constant  $1 \text{ ml min}^{-1}$  flow. Gradient chromatography was then carried out on a RP-HPLC Bischoff NPS C18 column ( $4.6 \text{ mm} \times 14 \text{ mm}$ ). Mobile phase A was 0.1% formic acid and mobile phase B was a mixture of acetonitrile:water:formic acid (900:100:0.1), a linear gradient of 0–100% B over 6 min being used. System control and data processing were provided by the Xcalibur program (Thermo Fisher Scientific).

### 3. Results

Human growth hormone periplasmic secretion in a bacterial host that had been transformed with the plasmids  $\lambda\text{P}_L$ -DsbA-hGH and pRK248cIts was studied at different activation temperatures, the qualitative and quantitative analysis of the hormone being carried out via RP-HPLC (Fig. 1). The  $\lambda\text{P}_L$  promoter is almost totally repressed up to  $37^\circ\text{C}$  as reported (Breitling et al., 1990), while at  $42^\circ\text{C}$  its derepression permits a hGH periplasmic secretion that is acceptable according to previously established parameters (Soares et al., 2003). Table 1 presents the results obtained in a set of different experiments, showing that without repressor a relatively high hGH secretion ( $1 \mu\text{g ml}^{-1}$ ;  $A_{600}^{-1}$ ) is obtained at  $30^\circ\text{C}$ , while an even higher secretion is obtained at  $42^\circ\text{C}$  either with or without repressor. Under the same conditions, for the expression vector in which

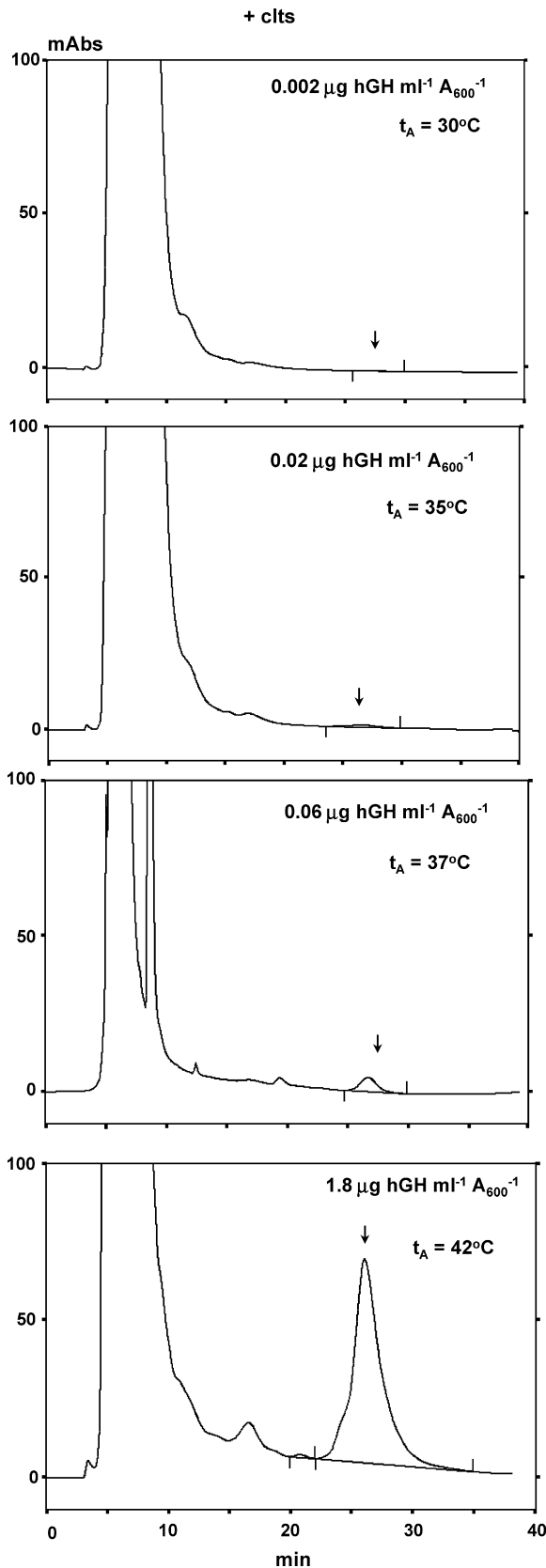


Fig. 1. Analysis of hGH periplasmic expression by isocratic RP-HPLC after activation at different temperatures, utilizing the hGH-producing W3110 *E. coli* strain transformed with pRK248cIts encoding for the repressor gene. The arrows indicate the peak of hGH.

Table 1

hGH periplasmic secretion level by “activation” at different temperatures ( $t_A$ ) utilizing hGH-secreting W3110 strains with or without the repressor gene (cIts)

$t_A$ ( $^\circ\text{C}$ )	$\lambda\text{P}_L\text{-DsbA-hGH (+ cIts)}$ ( $\mu\text{g ml}^{-1} A_{600}^{-1} \pm \text{S.D.}$ )	$\lambda\text{P}_L\text{-DsbA-hGH (- cIts)}$ ( $\mu\text{g ml}^{-1} A_{600}^{-1} \pm \text{S.D.}$ )
30	0.01	$1.0 \pm 0.14$ ( $n=2$ )
35	0.02	–
37	0.06	–
42	$1.31 \pm 0.38$ ( $n=4$ )	$1.61 \pm 0.11$ ( $n=3$ )

the hGH gene has been substituted by the hPRL gene and in the presence of cIts, an approximately 50-fold lower secretion had been observed at  $42^\circ\text{C}$  for this hormone. This led us to study better the behavior of our hPRL-producing *E. coli* strains, either transformed with pRK248cIts or not.

Since the bacterial periplasmic environment has been found to be quite detrimental to protein stability, especially above room temperature (Makrides, 1996; Simmons and Yansura, 1996), we carried out a study of hPRL periplasmic secretion by “activating” at different temperatures (range  $30\text{--}42^\circ\text{C}$ ), with and without the presence of the repressor. Human PRL secretion levels as a function of the temperature are illustrated in Fig. 2, while Fig. 3 shows qualitative and quantitative RP-HPLC analyses of the osmotic shock fluids obtained with both hPRL strains (+cIts and –cIts) under the described conditions. An “activation” temperature of  $37^\circ\text{C}$  and the bacterial strain lacking the cIts repressor thus provided the highest hPRL secretion level, i.e., approximately 30-fold higher than those obtained with the equivalent strain containing the repressor gene. Table 2 summarizes the statistical differences in the prolactin specific yields obtained at the different temperatures with or without the presence of the repressor. Even at  $42^\circ\text{C}$  the strain lacking the repressor gene produces a significantly higher ( $P < 0.001$ ) amount of hPRL when compared to the same condition in the presence of the repressor, a tendency also observed for hGH (Table 1).

Since it has been reported that the lack of repressor could easily lead to plasmid loss (Caulcott and Rhodes, 1986; Crowl, 1986), a study was carried out in shake flask cultures (LB medium) to determine the hPRL periplasmic yield in the strain

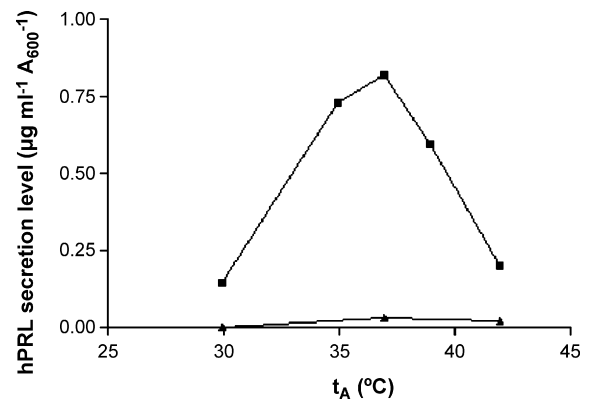


Fig. 2. hPRL expression level, determined by RP-HPLC, in osmotic shock fluids obtained under different “activation” temperatures, utilizing hPRL-secreting W3110 *E. coli* strain, either transformed or not with pRK248cIts (—■— without cIts; —▲— with cIts).

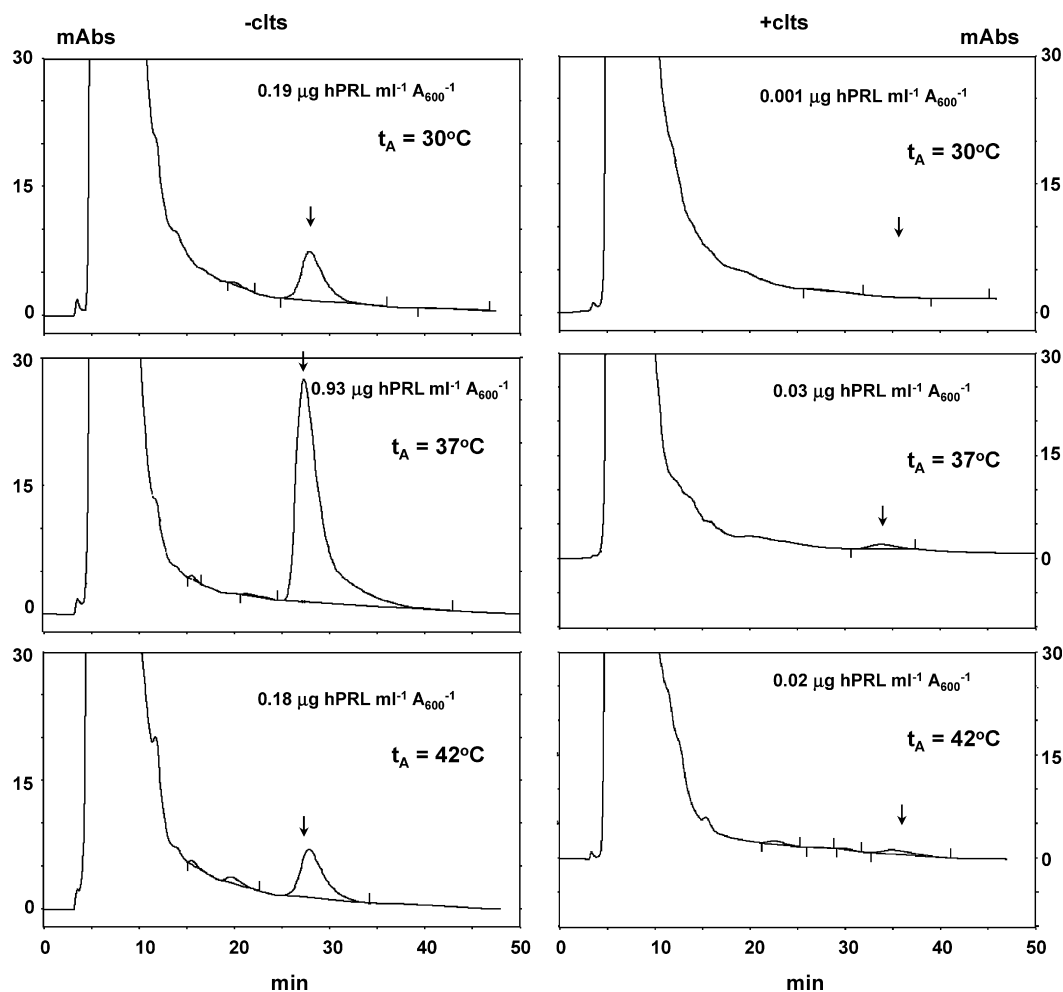


Fig. 3. Analysis of hPRL periplasmic expression by isocratic RP-HPLC after “activation” at different temperatures, utilizing the hPRL-producing W3110 *E. coli* strain either transformed or not with the plasmid pRK248cIts. The arrows indicate the peak of hPRL.

lacking cIts after two growth periods corresponding to 10 and 50 *E. coli* generations, obtaining practically the same specific expression level:  $0.64 \pm 0.05$  and  $0.78 \pm 0.03 \mu\text{g hPRL ml}^{-1} \text{A}_{600}^{-1}$ , respectively. In addition, the presence or absence of antibiotic (amp) did not influence the specific expression yield for at least 40 generations.

Table 3 compares results obtained with the hPRL-producing W3110 *E. coli* strain in a pilot scale bioreactor with those for a regular cultivation in shake flasks and with those for optimized fed batch culture of the analogous hGH-producing strain. Glycerol was also utilized as the carbon source instead of glu-

cose, as suggested by different authors (Jensen and Carlsen, 1990; Tabandeh et al., 2004), providing a good yield of biomass, but poor hPRL expression. In hPRL fed batch cultures, it was not possible to reach the same high biomass obtained for hGH. However, by activating at  $37^\circ\text{C}$  with low glucose addition during fed batch and activation ( $1.1 \text{ g l}^{-1} \text{ h}^{-1}$ ), it was possible to maintain good specific expression levels (up to  $1 \mu\text{g ml}^{-1} \text{A}_{600}^{-1}$ ) and an acceptable biomass ( $\text{A}_{600} = 35.3$ ) and production yield ( $\sim 35 \mu\text{g ml}^{-1}$ ). Also noteworthy is the demonstration that simple batch cultivation conditions, at  $37^\circ\text{C}$ , with no glucose addition as in experiment No. 5, can provide a useful yield of

Table 2

hPRL periplasmic secretion levels in *E. coli* W3110 by “activation” at different temperatures utilizing a strain containing (+ cIts) or not containing (– cIts) the repressor gene

$t_A$ ( $^\circ\text{C}$ )	$\lambda\text{P}_L\text{-DsbA-hPRL (+ cIts)}$ ( $\mu\text{g ml}^{-1} \text{A}_{600}^{-1} \pm \text{S.D.}$ )	$\lambda\text{P}_L\text{-DsbA-hPRL (- cIts)}$ ( $\mu\text{g ml}^{-1} \text{A}_{600}^{-1} \pm \text{S.D.}$ )	Statistical significance <sup>a</sup>
30	0.001 ( $n = 1$ )	$0.14 \pm 0.02$ ( $n = 6$ )	–
35	–	$0.73 \pm 0.07$ ( $n = 5$ )	$P < 0.001$
37	0.03 ( $n = 1$ )	$0.92 \pm 0.10$ ( $n = 6$ )	$P < 0.01$
39	–	$0.60 \pm 0.12$ ( $n = 8$ )	$P < 0.001$
42	0.02 ( $n = 1$ )	$0.19 \pm 0.05$ ( $n = 4$ )	$P < 0.001$

<sup>a</sup> Student's *t*-test comparing each value obtained with  $\lambda\text{P}_L\text{-DsbA-hPRL (- cIts)}$  to the previous one.

Table 3  
Cultivation yields, determined by RP-HPLC, of hPRL-producing W3110 *E. coli* strain in a pilot scale bioreactor in comparison with a regular shake flask culture and with an optimized cultivation of an analogous hGH-producing strain

Exp. no.	Process	Specific expression ( $\mu\text{g ml}^{-1} A_{600}^{-1} \pm \text{S.D.}$ )	OD ( $A_{600}$ )	Hormone yield ( $\mu\text{g l}^{-1}$ )	Process time (h)
1	hGH- producing strain (+cIts), fed batch culture (4× HKSII), with glucose, $t_A$ 42 °C	1.50	63.2	94.8	20
2	hPRL- producing strain (– cIts), optimized shake flask culture (LB), $t_A$ 37 °C	0.93	3.0	2.8	16
3	hPRL- producing strain (– cIts), same conditions as in Exp. 1, except $t_A$ 37 °C	0.17	42.4	7.2	20
4	Same conditions as in Exp. 3, but using glycerol instead of glucose (2× HKSII)	0.07	53.4	3.7	23
5	hPRL- producing strain (– cIts), batch culture (2× HKSII) with no glucose addition, $t_A$ : 37 °C	0.58	18.3	10.6	10
6	hPRL- producing strain (–cIts), fed batch (2× HKSII) with low glucose addition <sup>a</sup> , $t_A$ 37 °C	1.0	35.3	35.3	21
7	Same conditions as in Exp. 6, except $t_A$ 42 °C	0.23	32.1	7.4	18

<sup>a</sup> 1.1 g glucose  $\text{l}^{-1} \text{h}^{-1}$  during fed batch and “activation”.

about 10 mg hPRL  $\text{l}^{-1}$  in a process that requires only a 10 h cultivation. Experiment No. 7 confirmed that, even in the bioreactor, a  $t_A$  increase from 37 to 42 °C determined ~80% loss of hPRL expression as occurred in shake flask cultures.

Osmotic shock fluids, analyzed by HPSEC–RIA, showed the presence of ~75% of monomeric form of hPRL. The presence of the mostly monomeric form of hPRL was also confirmed by Western blot analysis, carried out on the total bacterial lysates and on the osmotic shock fluids, obtained at either 37 or 42 °C, in order to verify the possible lability of hPRL at higher temperatures (Fig. 4). Indeed, the bands of unmodified hPRL are much more intense at 37 °C and, in the lysate, there are considerably more degradation products at 42 °C. In contrast, purified pro-

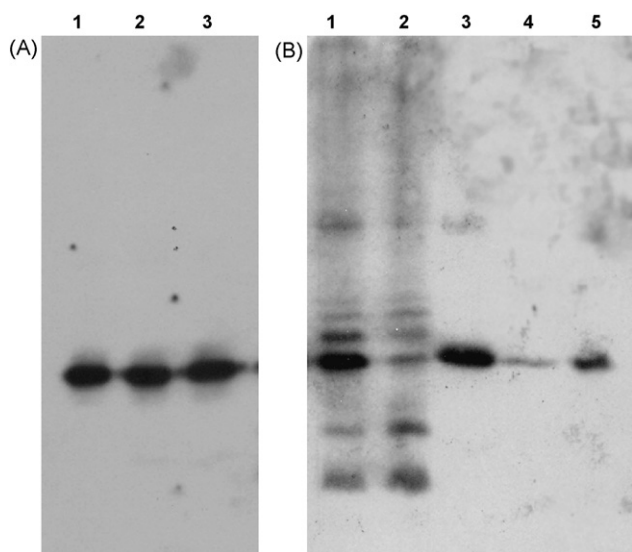


Fig. 4. Western blot analysis of *E. coli*-derived purified rhPRL, total bacterial lysates and osmotic shock fluids. (A) 40 ng of purified, rhPRL stored at –20 °C (1) or incubated for 10 h at 37 °C (2) or at 42 °C (3). (B) To each well (1–4) a sample derived from 1.5  $A_{600}$  units of bacterial mass was applied. Lysate of a sample of *E. coli* activated at 37 °C (1) or at 42 °C (2). Osmotic shock fluids obtained after activation at 37 °C (3) or at 42 °C (4). Purified rhPRL, stored at –20 °C (5).

lactin was found to be quite stable after 10 h incubation at either 37 or 42 °C, when compared to a sample kept at –20 °C.

The identity of purified hPRL was then confirmed by HPSEC, ESI-MS and *in vitro* bioassay in comparison with the International Chemical Reference Standard and with the First WHO Reference Reagent (97/714). Fig. 5 shows that the retention times ( $t_R$ ) of our preparation and hPRL-CRS (WHO) are prac-

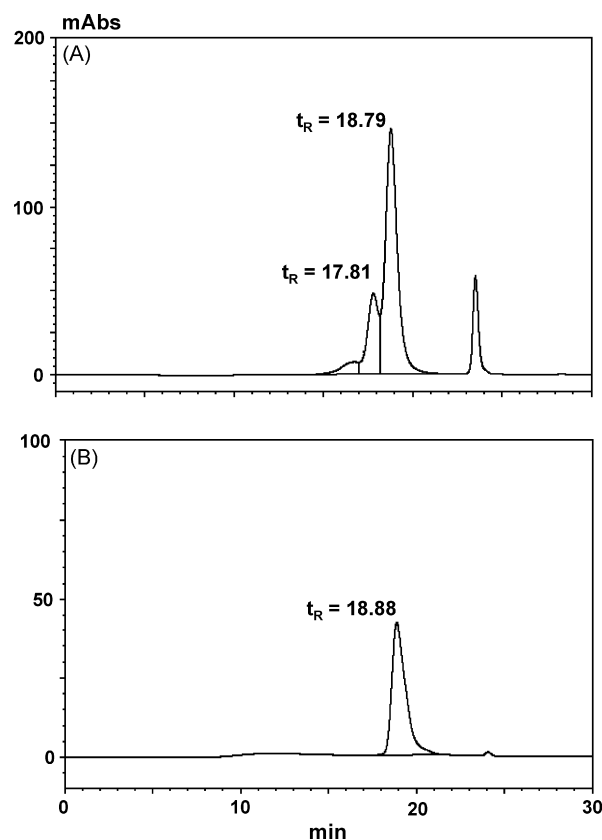


Fig. 5. Comparative HPSEC analysis of 11  $\mu\text{g}$  of hPRL-CRS (WHO) (A) and of 2.5  $\mu\text{g}$  of purified *E. coli* periplasmic hPRL, obtained in the present study (B) and applied to a Tosohaas G2000 SW column (60 cm  $\times$  7.5 mm i.d.). The WHO Reference Reagent of G-hPRL presented, in a parallel run, a  $t_R$  = 17.69 min.

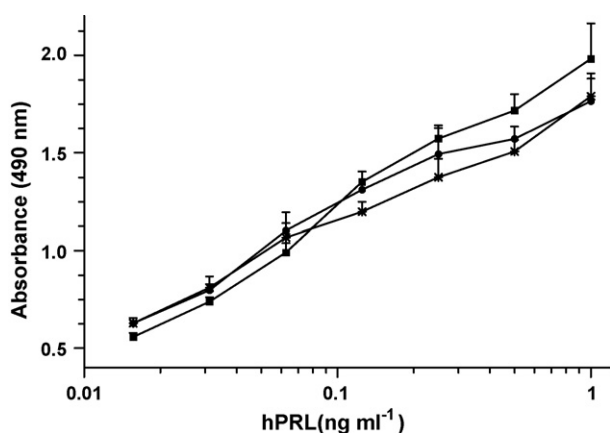


Fig. 6. Dose-response of Nb2 lymphoma cell cultures at 72 h to purified periplasmic hPRL (quantified by HPSEC against the WHO CRS) (□), the 1st WHO Reference Reagent 97/714 (57.2 IU mg<sup>-1</sup>) (■) and the WHO-CRS of recombinant hPRL (●).

tically identical. It is interesting to note the high purity of our preparation and the presence of ~21% of glycosylated hPRL ( $t_R = 17.81$ ) in the CRS, as detected in the same preparation during the International Collaborative Study promoted by NIBSC in 2000 (Rafferty et al., 2001). The fast running peak on the right of Fig. 5A is due to the excipient that is present in the reference preparation only. These data, moreover, confirm the good stability of a CRS solution, stored for ~6 years at  $-80^\circ\text{C}$ .

Our preparation of rhPRL, when analyzed by ESI-MS, provided a Mr of 22,905.8 against a Mr of 22,908.6 provided by the CRS of hPRL, both with a difference of less than 0.05% compared to the calculated molecular mass of hPRL (Wu et al., 2003). The same preparation, when analyzed via the Nb2 bioassay, indicated a mitogenic activity of  $51.5 \pm 24.1$  IU mg<sup>-1</sup> ( $n=3$  assays), a potency which is not significantly different ( $P>0.05$ ) from that ( $57.2 \pm 11.4$  IU mg<sup>-1</sup>,  $n=5$  assays) of the WHO Reference Reagent 97/714 (Fig. 6).

#### 4. Discussion

A relatively high secretion level (about  $1 \mu\text{g ml}^{-1} A_{600}^{-1}$ ), the highest yet reported for periplasmic hPRL, has been obtained by constitutive expression based on the unrepressed  $\lambda P_L$  promoter, at  $37^\circ\text{C}$ . The expression level is approximately 12-fold higher than that obtained in previous work (Morganti et al., 1998) by using an IPTG-activated tac promoter. This specific secretion yield is, however, still significantly lower ( $p<0.001$ ) than that observed when the hGH gene is inserted into the same expression vector, instead of hPRL. It has been demonstrated that hPRL periplasmic secretion, under control of the constitutive  $\lambda P_L$  promoter, has a maximal efficiency at  $37^\circ\text{C}$  (see Fig. 2), decreasing at higher temperatures. The dramatic decrease of secretion above  $37^\circ\text{C}$  has been attributed to the thermal lability of a heterologous protein and/or to an increased activity of proteases present in the periplasmic environment (Caulcott and Rhodes, 1986). As postulated by Lowman and Bina (1990) the levels of accumulated heterologous protein can be the result of an equilibrium between rate of protein synthesis and rate of degradation. For

hPRL such equilibrium could be particularly in favor of the rate of synthesis, at  $37^\circ\text{C}$ . As a matter of fact, an increased degradation due to proteolytic activity above an activating temperature of  $40^\circ\text{C}$  has been reported for human cystatin C (Dalbøge et al., 1989) and even for hGH (Tabandeh et al., 2004), a molecule that is considered to be more stable than hPRL. With basis on the results shown in Fig. 4, we could conclude that hPRL is highly degraded in the cytoplasm, at  $42^\circ\text{C}$ , a degradation that, to a limited extent, is already occurring at  $37^\circ\text{C}$ . This seems to be due to an increased activity of cytoplasmic proteases, because purified hPRL is stable at these temperatures.

According to the present study, the cIts repressor protein appears to function adequately up to  $37^\circ\text{C}$ , still exerting some negative influence on expression even at  $42^\circ\text{C}$ . This can be seen in both the case of hGH and of hPRL expression by comparing the specific expression yields at this temperature with or without the presence of cIts. We could also speculate that the higher expression at  $42^\circ\text{C}$  in systems lacking the repressor protein could be due to a certain protection against degradation offered by the periplasmic environment (Makrides, 1996) on the protein that had been previously synthesized during cultivation at  $30^\circ\text{C}$  (see Fig. 3). This appears to be confirmed by the fact that there is no evidence of degraded products in the periplasmic fluid (see Fig. 4).

The lack of repression (i.e., the use of a strain lacking pRK248cIts) apparently did not lead to plasmid loss of the type suggested by different authors (Bernard and Helinski, 1979; Crowl, 1986; Caulcott and Rhodes, 1986; Makrides, 1996) since there was no significant decrease in specific expression yield even after 40 generations. We must stress, though, that this experiment was carried out in shake flasks with LB medium and no glucose addition.

Experiments carried out in a pilot scale bioreactor have shown that the use of  $\lambda P_L$  as a constitutive promoter, a strict control of the temperature ( $37^\circ\text{C}$ ) and limited glucose addition ( $1.1 \text{ g l}^{-1} \text{ h}^{-1}$ ) can provide good specific expression levels and useful yields of hPRL. Addition of the carbon source in higher amounts, in order to reach higher biomass, always led to very low hPRL expression. It is difficult to anticipate now the future demands for hPRL, especially for therapeutic applications. Given yields of  $\sim 30 \text{ mg l}^{-1}$  of authentic hormone (see Table 3), and considering a purification recovery of  $\sim 40\%$ , a 150 l bioreactor (the same we have been using for relatively small hGH productions) could provide  $\sim 1.5 \text{ g}$  of purified hormone per fermentation process. Thus, together with the relatively short fermentation time ( $\sim 20 \text{ h}$ ), a competitive yearly production of up to  $>200 \text{ g}$  of purified hPRL should be possible even on this scale.

The data presented here also confirm the identity, proper folding and bioactivity of hPRL obtained via our cultivation and purification protocol. HPSEC, carried out on a particularly long chromatographic column that permits detection of minor structural alterations of the protein molecule (Barth et al., 1998; Mendonca et al., 2005; Ribela et al., 2006), confirmed the presence of a correctly processed monomeric form, whose chromatographic behavior was practically identical to that of the International Reference Reagent. An analogous agreement

was also demonstrated via ESI-MS and the Nb2 *in vitro* bioassay. Moreover, an analogous preparation of *E. coli*-derived hPRL exhibited, in previous work, the practically identical Mr of 22888 (Soares et al., 2006).

In conclusion, the present results clearly demonstrate the feasibility of utilizing *E. coli* instead of mammalian or insect cell expression systems (Price et al., 1995; Hirooka et al., 1996; Soares et al., 2000; Das et al., 2000; Pereira et al., 2001) for the efficient and economic production of authentic and highly homogeneous hPRL.

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