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Periplasmic expression of human growth hormone via plasmid vectors containing the λP_L promoter: use of HPLC for product quantification

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The influence of different factors acting on *Escherichia coli* periplasmic expression of recombinant human growth hormone (hGH) in shake flask cultures has been investigated. Bacterial vectors containing the phage λP_L promoter, which is temperature activated, were utilized. Four different signal peptides were compared: DsbA, npr, STII and one derived from the natural hGH signal peptide, this last used as a reference. Other factors such as medium composition, optimized induction and expression conditions, and different bacterial strains were also studied. The determination of hGH, carried out directly in osmotic shock fluids, was based on an isocratic reversed-phase high-performance liquid chromatography method, which allows direct, rapid evaluation of the quality and quantity of hGH being secreted in the bacterial periplasmic space immediately after or even during fermentation. The level of hGH production increased 2.5-fold compared with the reference vector, reaching a level of 3.9 ± 0.63 μg/ml/A_{600} (n = 6; coefficient of variation = 16.2%). The expression level was affected by the signal peptide and by the induction conditions, being more effective when activation started in the early logarithmic phase which, however, exhibited remarkably different optical density (OD) according to medium composition. Our results thus indicate that 6 h activation at 40–42°C, starting with an OD (A_{600}) of ~3 in a very rich medium, were conditions capable of providing the maximum secretion level for a vector utilizing the DsbA signal sequence and *E.coli* W3110 or RB791 as host cells.

Keywords: *Escherichia coli* periplasm/human growth hormone/λP_L promoter/recombinant DNA/signal peptide

Introduction

Human growth hormone (hGH), a polypeptide of 22 125 Da (191 amino acids), is synthesized in the anterior pituitary gland. Owing to its variety of biological activities, hGH has therapeutic applications in the treatment of children with growth hormone deficiency and chronic renal failure, girls with Turner syndrome, adults with growth hormone deficiency or HIV wasting syndrome (Tritos and Mantzoros, 1998). In addition, applications in the treatment of bone fractures, skin burns and bleeding ulcers have been suggested. Its use in other conditions such as children with idiopathic short stature, the healthy elderly and the critically ill is also being investigated (Shin *et al*., 1998; Tritos and Mantzoros, 1998; Finkelstein *et al*., 2002; Wit, 2002).

The major form of hGH (22 kDa) is synthesized from the hGH-N gene in the non-glycosylated form (Garcia-Barros *et al*., 2000), so prokaryotic expression systems such as *Escherichia coli* have been the choice host cells for the production of this recombinant protein. High-level expression of hGH has been obtained by using procedures known to optimize both gene transcription and mRNA translation and several groups have reported the expression of the hormone or of a derived fusion protein in *E.coli* cytoplasm (Goeddel *et al*., 1979; Ikehara *et al*., 1984; Dalböge *et al*., 1987; Mukhija *et al*., 1995; Shin *et al*., 1998; Patra *et al*., 2000). However, the cytoplasmic production of a protein has certain disadvantages: high-level accumulation often leads to insoluble protein aggregates that can be difficult to solubilize; a refolding step is frequently required to obtain the native conformation and to form the correct disulfide bonds; the protein of interest usually contains an N-terminal methionine (Becker and Hsiung, 1986) that may play a role in antibody formation in patients treated with the hormone (Glasbrenner, 1986). Alternative expression systems have been based on the secretion of the protein into the *E.coli* periplasmic space; in these systems, the protein is obtained in its soluble and perfectly folded form and can be more easily purified to homogeneity. Secretion, which mimics the natural process of the somatotropic cells in the pituitary gland, was achieved by linking the signal peptide sequence to the hGH gene. All signal sequences, composed of 15–30 amino acids, are characterized by several charged amino acids at the N-terminus, hydrophobic amino acids in the central region and an amino acid with a short side chain at the cleavage site. It has been demonstrated that the signal sequence functions to facilitate the translocation of secretory proteins across the inner membrane. A membrane-bound signal peptidase then cleaves off the signal sequence to release the mature protein into the periplasmic space located between the inner and outer membranes of *E.coli* (Chang *et al*., 1987).

Table I summarizes the most noteworthy periplasmic expression yields reported in the literature for hGH, together with the corresponding references and the analytical method utilized for the hormone determination. Except for our group, hGH quantification was performed by either RIA or ELISA, which has quite limited accuracy, especially in the case of complex mixtures like culture broth and crude extracts (Jacobson *et al*., 1997; De Oliveira *et al*., 1999). Immunoassays, moreover, cannot discriminate between undesired hGH-related forms (e.g. mass and charge isomers) and the fundamental form of the hormone. Such discrimination is, however, now possible when one uses the more recently developed HPLC or free zone capillary electrophoresis (FZCE) methodologies that can also be directly applied to *E.coli* fermentation broth for both qualitative and quantitative purposes (Strege and Lagu, 1995; McNerney *et al*., 1996;

Table I. Reported periplasmic expression of recombinant hGH in *E.coli*

hGH expression level (μg/ml/OD)	Host cell	Promoter	Signal peptide	Analytical method for hGH determination	Reference
1.5	*P.aeruginosa*	trp	hGH	RIA	Gray *et al*., 1984
0.6	*E.coli* 294	trp	phoA	RIA	Gray *et al*., 1985
10–15	*E.coli* K12RV308	Lpp-lac	ompA	RIA/ELISA	Becker and Hsiung, 1986; Hsiung *et al*., 1989
15–25	*E.coli* W3110	phoA	STII	RIA	Chang *et al*., 1987
11.2[a]	*E.coli* HB101	Penicillinase	Penicillinase	RIA	Kato *et al*., 1987[b]
10–15	*E.coli* BL21	trc	Omp A	RIA	Cheah *et al*., 1994
10	*E.coli* W3110	npr	npr	ELISA	Uchida *et al*., 1997
0.8	*E.coli* RRI	λP_L	hGH	RP-HPLC	Dalmora *et al*., 1997

[a]μg/ml of culture medium.
[b]hGH was transported through the periplasm and excreted into the medium.

Dalmora *et al*., 1997; Jacobson *et al*., 1997; Jorgensen *et al*., 1998; Karlsson *et al*., 1999; Ueda *et al*., 2001; Soares *et al*., 2002; Ribela *et al*., 2003).

In previous studies, we reported the construction and use of an *E.coli* expression vector (Ribela *et al*., 1993; De Oliveira *et al*., 1999) in which the gene encoding for hGH was under the control of the λP_L promoter and included a signal sequence derived from the natural hGH gene. In the present study, we report results for other expression vectors based on different signal peptides. These are DsbA (Martin *et al*., 1993) and STII (Picken *et al*., 1983) from *E.coli* and modified npr (Uchida *et al*., 1997) from *Bacillus amyloliquafaciens*. Other factors that influence the expression level, such as stage, length and temperature of induction, medium composition and host strain, have also been investigated in order to find the optimum conditions for a high-level periplasmic hGH secretion. This study was carried out in shake flask cultures (Erlenmeyer flasks) rather than in a laboratory bioreactor of the type used previously (Dalmora *et al*., 1997; Oliveira *et al*., 1999) in order to develop a methodology that allowed quick and easy comparison of the expression level of several bacterial strains harboring different vectors under different conditions, before starting the real process set up in a bioreactor.

Materials and methods

Plasmid constructions

A cassette expression vector was obtained by introduction of the restriction endonucleases sites *Nde*I upstream of the starting codon by polymerase chain reaction (PCR), using as template a previously constructed expression vector (our reference vector) in which the hGH gene is preceded by a modified natural hGH signal peptide.

The synthetic primers used for this PCR were: forward primer, including the *Nde*I site and 6 nt of the hGH signal peptide (5′-GAG GAA TTC CAT ATG GGC TCC-3′); reverse primer, including a C-terminal sequence of the hGH gene and the *Bam*HI site, downstream of the stop codon (5′-ATC GGA TCC TTA TCA GAA GCC ACA GCT GCC CTC C-3′).

For the introduction of three different signal sequences the following synthetic primers were used. (i) DsbA–hGH sequence: forward primer, including the *Nde*I site, 57 nucleotides of the DsbA signal peptide (underlined) and part of the hGH gene (5′-GAG GAA TTC CAT ATG AAA AAG ATT TGG CTG GCG CTG GCT GGT TTA GTT TTA GCG TTT AGC GCA TCG GCG TTC CCA ACC ATT CCC TTA TCC-3′); reverse primer, the same used for cassette expression vector construction. (ii) STII–hGH sequence: forward primer, including the *Nde*I site, 69 nucleotides of the STII signal peptide (underlined) and part of the hGH gene (5′-GAA TTC CAT ATG AAA AAG AAT ATC GCA TTT CTT CTT GCA TCT ATG TTC GTT TTT TCT ATT GCT ACA AAT GCC TAT GCA TTC CCA ACC ATT CCC TTA TCC-3′); reverse primer, the same used for cassette expression vector construction. (iii) npr2K5L–hGH sequence: this was obtained with two PCRs. Reaction 1: synthesis of the forward primer utilized for reaction 2. Forward primer (5′-GAG GAA TTC CAT ATG GGT TTA GGT TTA GGT AAG AAA AAG AAA TTG TTA CTT CTC CTA CTG TCT AGT GCT GTC GCC GCT TCC TTT ATG-3′); reverse primer (5′-GGA TAA GGG AAT GGT TGG GAA GGC CTG AAC ACC CGG CAG ACT GAT GGT TAA ACT CAT AAA GGA AGC GGC GAC AGC-3′). Reaction 2: synthesis of the complete sequence npr2K5L–hGH. Forward primer (product of reaction 1), including the *Nde*I site, 102 nucleotides of the npr2K5L signal peptide (underlined) and part of the hGH gene (5′-GAG GAA TTC CAT ATG GGT TTA GGT TTA GGT AAG AAA AAG AAA TTG TTA CTT CTC CTA CTG TCT AGT GCT GTC GCC GCT TCC TTT ATG GCT GTC GCC GCT TCC TTT ATG AGT TTA ACC ATC AGT CTG CCG GGT GTT CAG GCC TTC CCA ACC ATT CCC TTA TCC-3′); reverse primer, the same used for cassette expression vector construction.

After digestion of the cassette expression vector and the DNA fragments obtained with each PCR with the endonucleases *Nde*I and *Bam*HI, the fragments were subcloned into the *Nde*I–*Bam*HI sites of the cassette, the resulting vectors being named DsbA–hGH, npr2K5L–hGH and STII–hGH (Maniatis *et al*., 1989).

Bacterial strains and cultivation

Escherichia coli RRI strain, carrying the low copy-number plasmid pRK248cIts encoding for the λP_L promoter repressor cI, was used as a primary host for transformation and propagation of plasmids. For hGH expression, the host strains W3110, HB2151, RB791, TP2339 and BMH7118 were also used. The last two strains were kindly donated by Sanofi-Synthélabo Recherche (Toulouse, France). The cultivation was carried out in 500 ml shake flasks containing 100 ml of Luria–Bertani (LB) medium, which consisted of yeast extract (5 g/l), tryptone (10 g/l) and sodium chloride (10 g/l), or a richer culture medium (2x-HKSII) which was a 2-fold concentrate of the HKSII bioreactor medium utilized by Jensen and Carlsen

(Jensen and Carlsen, 1990), consisting of yeast extract (10 g/l), tryptone (20 g/l), acid hydrolyzed casein (4 g/l), salts (Mg, K and Ca) and trace metals (Fe, Zn, Mn, Cu, Co, B, Mo and I). Both media were supplemented with 100 µg ampicillin/ml. Each flask was inoculated with a single colony of *E.coli* cells harboring one of the described hGH expression vectors taken from LB agar plates and incubated on a rotary shaker at 30°C. After growth to different optical density (OD) levels, the culture was either maintained at 30°C or heat-induced at different temperatures as described.

Osmotic shock

Periplasmic osmotic shock fluid was obtained by the method of Koshland and Botstein (Koshland and Botstein, 1980). Briefly, a volume of fermentation broth corresponding to 100 A_{600} was harvested 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 1.0 ml of ice-cold 10 mM Tris–HCl, pH 7.5, containing 20% (w/v) sucrose. Then, 33 µl of 0.5 M EDTA, pH 8.0, were added and incubation on ice continued for 10 min. The cells were then centrifuged and the pellet rapidly resuspended by vigorous agitation in 1.0 ml of a cold 1 mM Tris–HCl, pH 7.5, solution. The suspension was then incubated for 10 min on ice and centrifuged again for 5 min. The supernatant was removed and saved as the periplasmic fraction, also called osmotic shock fluid.

Analytical reversed-phase high-performance liquid chromatography (RP-HPLC)

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. The column was a C4 Vydac 214TP54 (25 cm×4.6 mm i.d., pore diameter of 300 Å and particle diameter of 5 µm) with a guard column (Vydac 214FSK54) between the sample injector and the main column and a silica precolumn packed with LiChrosorb Si-60, 7.9–12.4 µm (Merck, Darmstadt, Germany) located between the pump and the injector. All Vydac columns were purchased from Grace Vydac (Hesperia, CA, USA). The mobile phase consisted of 71% Tris–HCl buffer (50 mM, pH 7.5) and 29% *n*-propanol, as described by Dalmora *et al.* (Dalmora *et al*., 1997), with a flow-rate of 0.5 ml/min, detector wavelength at 220 nm, column temperature maintained at 45°C and a sample volume of 25–200 µl.

SDS–PAGE

Discontinuous SDS–PAGE, based on 15% polyacrylamide gels, was carried out under non-reducing conditions as described (Laemmli, 1970). Coomassie Brilliant Blue G-250 (USB, Cleveland, OH) was used for staining and the molecular mass markers were from Amersham Pharmacia Biotech (Piscataway, NJ).

Protein determination

The total protein present in the osmotic shock fluids was determined by a Micro BCA™ Protein Assay Reagent Kit (Pierce, Rockford, IL), based on a detergent-compatible bicinchoninic acid formulation for the colorimetric detection and quantification of total protein in dilute aqueous solutions.

Results

Duration of activation period

The influence of the length of the activation period on the hGH expression level was investigated in Erlenmeyer flask culture as shown in Figure 1. *Escherichia coli* RRI cells harboring the reference vector were grown in LB medium at 30°C. At an OD of A_{600} = 1.5, the temperature was increased to 42°C. The cell density value for starting activation was defined by preliminary experiments, then confirmed by the results presented below. After activation periods of 0, 2, 4, 6, 7 and 8 h, samples were withdrawn and the osmotic shock fluid was analyzed by RP-HPLC.

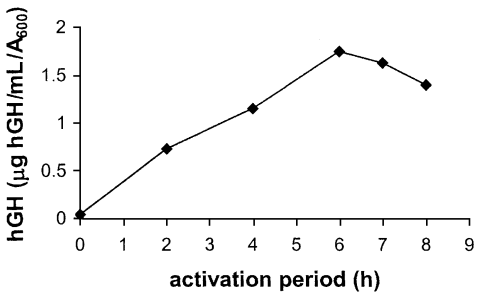

Fig. 1. hGH expression level, determined by isocratic RP-HPLC, in osmotic shocks fluids obtained after different activation periods of an *E.coli* RRI strain transformed with the reference vector.

The expression level of hGH increased during activation, reaching a maximum after 6 h. Longer activation periods, above 8 h, produced a significant increase of altered forms and/or a lower hGH expression.

Stage of induction

In order to examine the effect of induction at different cell densities on hGH expression, the *E.coli* RRI strain transformed with the reference vector was incubated at 30°C in LB medium. When the cell density reached ODs of 0.3, 0.5, 1.0 or 1.5, the temperature was increased to 42°C. After a 6 h activation period, the levels of hGH expression at each induction stage were compared, as shown in Figure 2. The expression levels progressively increased from 0.4 (activating at A_{600} = 0.3) to 1.4 µg/ml/A_{600} (activating at A_{600} = 1.5), but decreased significantly above A_{600} = 2.0 (data not shown). In the case of activation at low cell density, besides obtaining a lower expression, a higher amount of altered forms could also be seen upon RP-HPLC analysis. The two growth curves as presented in Figure 3 illustrate the different behavior occurring when the richer medium 2x-HKSII is used. In the latter case, the best expression was obtained upon activaton at A_{600} = 3.0 (data not shown).

Activation temperature

In order to examine the effect of the temperature of activation on hGH expression, the reference vector in the *E.coli* RRI strain, was incubated in either LB or 2x-HKSII medium at 30°C. The activation in the latter medium was initiated at A_{600} = 3.0, utilizing different temperatures between 30 and 45°C (Figure 4).

The maximum expression level of hGH was observed at 40–42°C, a dramatically decreased expression being observed at higher activation temperatures. The temperature of activation of 41°C was therefore confirmed as the most convenient for our systems.

Comparison between different media and with established bioreactor fermentation conditions

hGH expression, using the reference strain and the optimized conditions, was analyzed in LB medium or in the same richer

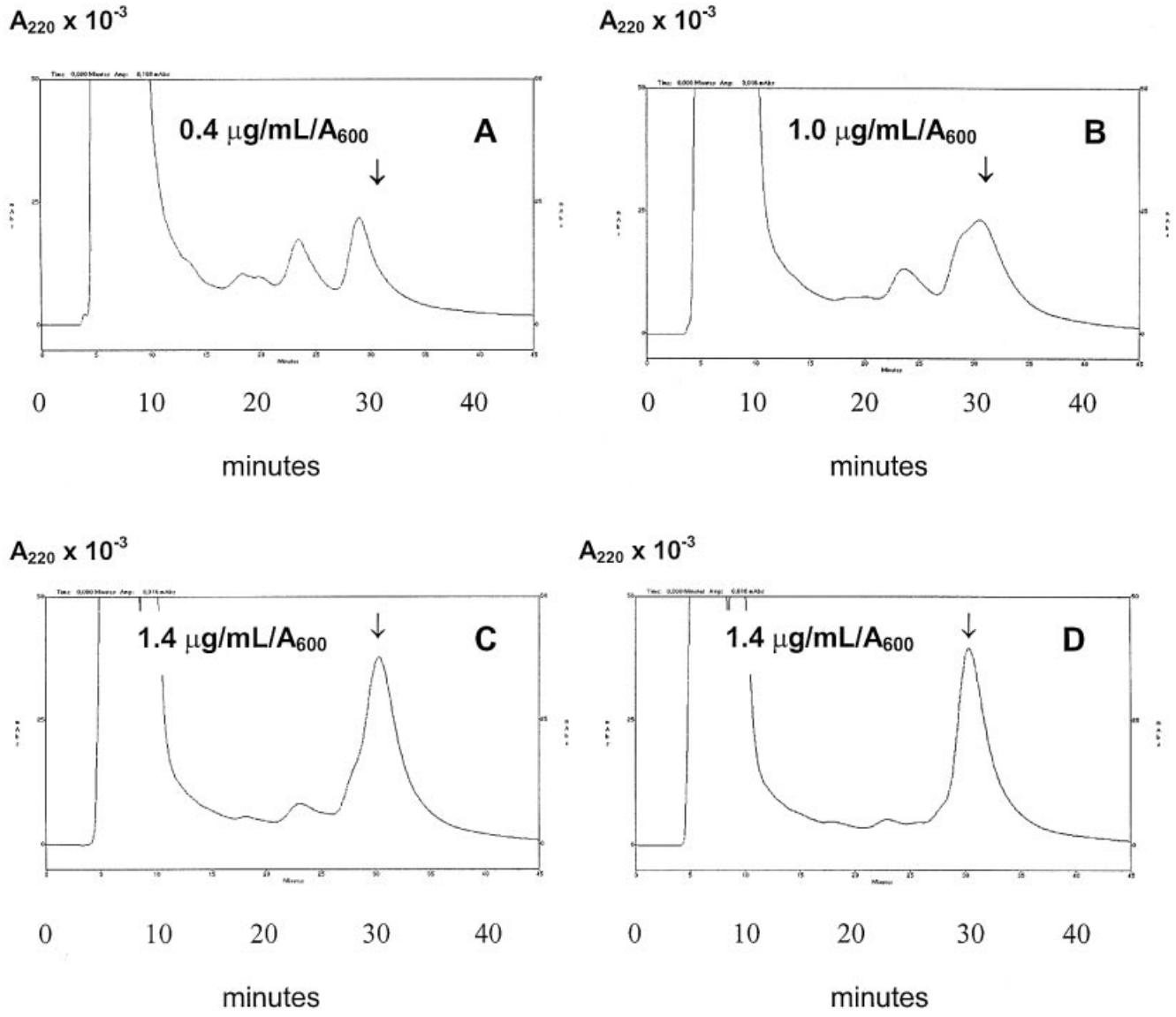

Fig. 2. Analysis by isocratic RP-HPLC of hGH expression after induction at different cell densities and incubation at 42°C for 6 h. (**A**) Activation at A_{600} = 0.3. (**B**) Activation at A_{600} = 0.5. (**C**) Activation at A_{600} = 1.0. (**D**) Activation at A_{600} = 1.5. Cell-specific hGH expression levels are reported in each figure. The arrows indicate the retention time (t_R) of the International Standard of hGH, run in the same experiment.

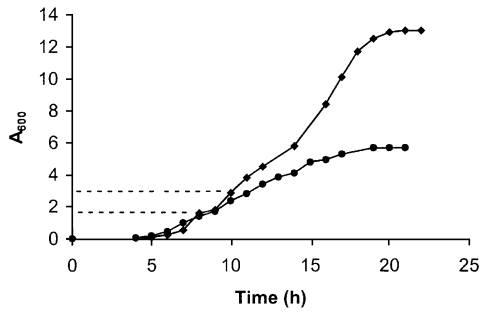

Fig. 3. Regular growth curves, without induction, of *E.coli*, RRI strain, at 30°C in LB (circles) and 2x-HKSII (diamonds) medium. The dotted lines indicate the ODs at which induction was carried out in the activation studies.

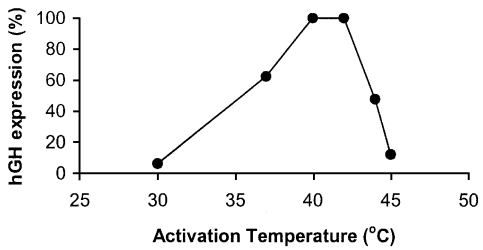

Fig. 4. hGH expression level, determined by RP-HPLC in osmotic shock fluids obtained under different activation temperatures.

2x-HKSII medium described by Jensen and Carlsen (Jensen and Carlsen, 1990) that we used for bioreactor studies (De Oliveira *et al*., 1999).

The cell-specific yield of hGH was practically the same in both media. Thus, with LB we obtained inter-assay statistics of 1.63 ± 0.65 μg/ml/A_{600} [coefficient of variation (CV) = 40%; n = 14], while with 2x-HKSII we obtained 1.56 ± 0.34 μg/ml/A_{600} (CV = 22%; n = 8). The main difference was in the growth curve, the final cell density after 6 h activation being almost 2-fold higher in 2x-HKSII medium: A_{600} = 6.1 ± 0.85 versus A_{600} = 3.5 ± 0.66. It is interesting to note that, in previous studies carried out in a 20 l laboratory bioreactor under controlled

Table II. Influence of different signal peptides on hGH secretion by *E.coli* RRI strain, in 2x-HKSII medium

	Signal peptide	hGH secretion level (μg/ml/A_{600})	*n*	Significance test[b]
hGH	Reference vector	1.56 ± 21.8[a]	9	–
	Cassette expression vector	1.80 ± 28.3	9	NS
DsbA		2.80 ± 23.0	21	$P < 0.001$
npr2K5L		2.20 ± 27.0	7	$P < 0.001$
STII		0.47 ± 84.0	3	$P < 0.001$

[a]CV, expressed as percentage of the mean.
[b]Student's *t*-test, in comparison with the reference vector.

fermentation conditions and glucose feed (De Oliveira *et al*., 1999), the same reference strain provided 2.43 ± 1.09 μg hGH/ml/A_{600} (CV = 45%; $n = 6$), i.e. an expression level ~50% higher when compared with Erlenmeyer cultivation in the same medium. It was later observed that the addition of 6 g/l glucose to the rich 2x-HKSII medium in Erlenmeyer flask culture practically inhibited all hGH expression, which went down to 0.07 μg/ml/A_{600}.

Effect of different signal peptides on hGH expression

To examine the influence of the signal peptide on hGH secretion into *E.coli* periplasm, we constructed four vectors using different signal peptides. Our reference vector, as mentioned, is based on a eukaryotic signal peptide, derived from the natural sequence of the hGH gene. The second signal peptide utilized was from DsbA, a 21 kDa periplasm protein of *E.coli* which, because of its origin and function, should provide an efficient periplasmic secretion (Martin *et al*., 1993). The third was the npr signal peptide, modified by inserting a 2K5L cluster into the N-terminal portion to enhance positive charge and hydrophobicity: this same modification caused increased secretion of 20K hGH in *E.coli* (Uchida *et al*., 1987). The fourth signal peptide, heat-stable enterotoxin II (STII), is also from an *E.coli* protein and has already provided a highly efficient periplasmic secretion of hGH (Chang *et al*., 1987). The hGH expression obtained with the four different signal peptides, using RRI as the host cell, is shown in Table II. The results show that the most efficient signal peptide–hGH gene combination was that based on DsbA, with an ~80% higher expression level compared with the reference vector.

The signal peptide influence, its correct processing and the efficient osmotic shock procedure were also confirmed by SDS–PAGE analysis (Figure 5). This figure shows the correct signal peptide cleavage, always producing authentic hGH with the same molecular weight as the hGH International Standard. It is also confirmed that DsbA is the most efficient signal peptide and that practically all hGH present in the periplasmic space was extracted.

Host cell comparison

Since *E.coli* strains differ in their ability to promote the expression of cloned genes (Kaytes *et al*., 1986), the most efficient vector, λP_LDsbAhGH amplified in RRI, was also used to transform the following strains: HB2151, W3110, RB791, TP2339 and BMH7118, their growth before activation being carried out at 30°C.

Activation was carried out, as already established, at a cell density of 3.0 A_{600} in 2x-HKSII medium. The expression levels obtained in *E.coli* W3110 and RB791 were significantly higher

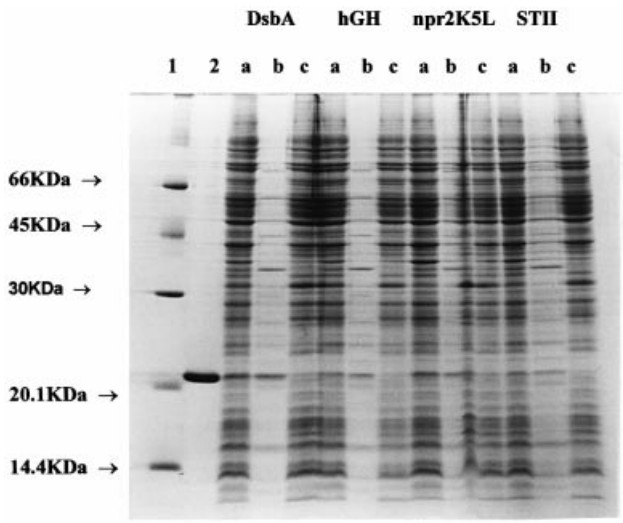

Fig. 5. SDS–PAGE analysis, under non-reducing conditions, of hGH periplasmic secretion. Four signal peptides were analyzed: DsbA, natural hGH, npr2K5L and STII. Lane 1, molecular-mass marker. Lane 2, International Standard of rec–hGH (WHO 98/574) (5 μg). a, proteins derived from total cell lysate incubated at 42°C for 6 h; b, periplasmic proteins extracted by the osmotic shock procedure; c, cytoplasmic proteins. Total cell lysates were prepared by centrifuging an aliquot of cell culture, adding SDS sample buffer to the cell pellet and boiling for 10 min. An amount of material derived from 0.25 A_{600} was loaded into each of the a, b and c wells.

Table III. Analysis of hGH secretion in the periplasm of various hosts, always utilizing the λP_L DsbA–hGH expression vector

Strain	hGH expression level (μg/ml/A_{600})	*n*	Total periplasmic hGH (μg/ml)	Total periplasmic protein (μg/ml)	hGH mass fraction (%)	Significance test[b]
RRI	2.8 ± 23.0[a]	21	290	1784	16.3	–
HB2151	1.2 ± 32.0	4	120	2107	5.7	$P < 0.001$
RB791	3.9 ± 8.1	2	420	2587	16.2	$P < 0.05$
W3110	3.9 ± 16.3	6	390	1990	19.6	$P < 0.005$
BMH7118	1.2 ± 9.4	3	120	1637	7.3	$P < 0.001$
TP2339	0.04 ± 15.7	4	40	920	0.43	$P < 0.001$

[a]CV, expressed as percentage of the mean.
[b]Student's *t*-test, comparing expression levels with that of the reference strain (RRI), transformed with the same vector (DsbA–hGH).

than those obtained using the same vector in RRI and 2.5-fold higher compared with the reference vector in RRI (Table III). It is also interesting to observe that W3110 was not only one of the most productive strains, but also provided the highest hGH mass fraction.

Figure 6 nicely illustrates, by RP-HPLC, the quality of the hGH and the secretion efficiency obtained in W3110 harboring the DsbA–hGH vector as compared with the product of the reference vector in RRI. Note that the sensitivity and vertical scale are the same for all three chromatograms.

Discussion

The highest hGH periplasmic secretion ever reported with a system based on the thermally inducible λP_L promoter, one of the most widely used for large-scale protein production since its use is simple, safe and cost-effective (Makrides, 1996), has

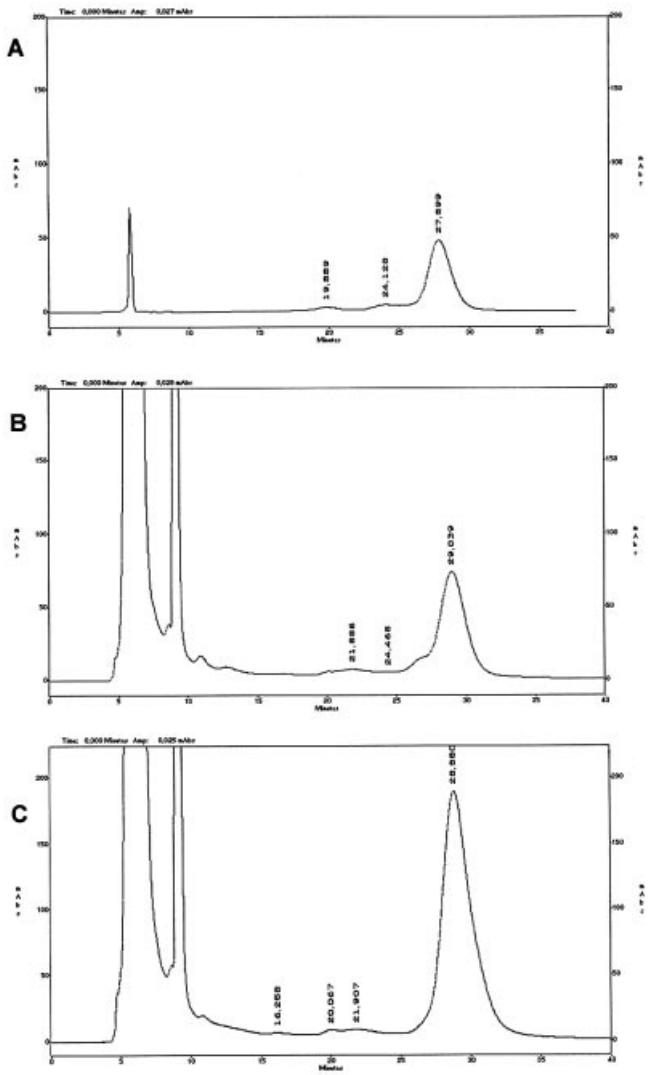

Fig. 6. Isocratic RP-HPLC analysis. (**A**) International Standard of hGH (10 µg). (**B**) Periplasmic proteins obtained using the reference vector in RRI (derived from 10 A_{600}). (**C**) Periplasmic proteins obtained using DsbA–hGH in W3110 (derived from 10 A_{600}).

been obtained by carefully optimizing the induction and expression conditions. Higher periplasmic expressions (up to 10–25 µg hGH/ml/OD) were reported by several authors (Hsiung *et al*., 1986; Chang *et al*., 1987; Cheah *et al*., 1994; Uchida *et al*., 1997) employing Lpp-lac, phoA, trc or npr promoters. In these cases, however, hGH quantification was based entirely on immunoassay methods that, as already mentioned, do not discriminate between the different isoforms of the hormone and, especially in complex mixtures, do not offer the same accuracy as a physico-chemical method such as RP-HPLC, which also permits qualitative and/or quantitative hGH determination directly in *E.coli* osmotic shock fluids (Strege and Lagu, 1995; Dalmora *et al*., 1997; Jacobson *et al*., 1997; De Oliveira *et al*., 1999).

It is important to emphasize that the present study was carried out in shake flask cultures, a widely used laboratory procedure that, unlike bioreactors, seldom has been standardized for this purpose. When expressing different recombinant proteins in *E.coli* under the control of a thermally inducible promoter, numerous authors have recommended starting the activation (i.e. induction stage) at an OD between 0.2 and 1.2, without a description of the optimization procedure leading to this choice. The same considerations also apply to the reported range of the temperature of growth (from 28 to 32°C) and activation (from 36 to 45°C) and to the duration of the activation period of 1–4 h (Bernard and Helinski, 1979; Crowl *et al*., 1985; Caulcott and Rhodes, 1986; Crowl, 1986; Breitling *et al*., 1990; Lowman and Bina, 1990; Cheng and Patterson, 1992; Balakrishnan *et al*., 1994; Love *et al*., 1996; Heim *et al*., 1998; Hoffmann *et al*., 1999; Yin *et al*., 2003).

In our study, the optimized period of activation found for shake flask cultures was 6 h, a result that is consistent with the observations of Simons *et al*. (Simons *et al*., 1984), Lowman and Bina (Lowman and Bina, 1990) and Choi and Lee (Choi and Lee, 1997) in their optimization of large-scale production of bovine growth hormone (bGH). The same optimum activation time was obtained by us in previous work employing the same reference vector for hGH expression, in which feed batch fermentation conditions were set up in a 20 l laboratory bioreactor (Dalmora *et al*., 1997). Although it may be possible to greatly increase biomass by up to 10–20-fold in a bioreactor under more controlled cultivation conditions (Makrides, 1996), an increase of this order does not occur for the protein of interest. In our bioreactor, the cell-specific yield of hGH was only ~50% higher than in shake flasks, with approximately the same inter-assay reproducibility.

Under our conditions, and in contrast to literature data, the optimized induction stage corresponded to A_{600} = 1.5 in the case of LB medium and to A_{600} = 3.0 for 2x-HKSII medium. In the case of LB medium, when activation was started below A_{600} = 1.0, a much lower yield of hGH (partly altered) was obtained, while above A_{600} = 2.0, the secretion yield also rapidly decreased. A similar situation was observed for the richer medium, though at higher A_{600} values. Figure 3 shows that the optimized stage of induction falls in the early log phase for both curves, as several authors recommend (Choi and Lee, 1997). This, however, corresponds to quite different OD values for the two media and obviously should be considered in any optimization process. Preliminary studies were also carried out with the use of our reference strain devoid of the plasmid encoding for the CI repressor but, at least up to 50 generations, no significant decrease in hGH expression was observed.

Concerning the temperature of activation, our data are in good agreement with the generally recommended literature value of 42°C. Above this temperature, hGH secretion yields decrease dramatically. Thus, to guarantee an acceptable inter-assay reproducibility, we preferred to set this parameter at 41°C to avoid losses as a result of occasional uncontrollable small temperature fluctuations. In the case of periplasmic prolactin (a much more labile protein hormone than hGH) for example, the activation temperature cannot exceed 38°C if good recoveries are to be obtained (data not shown). Quite interesting in this respect is the approach of several authors who describe activation at 42–45°C for a short time (e.g. 5–20 min) followed by incubation for a longer period at 37–41°C (Bernard and Helinski, 1979; Caulcott and Rhodes, 1986; Balakrishnan *et al*., 1994). There have been several reports, however, suggesting that induction temperatures below 42°C may be more suitable for protein production in general (Lowman and Bina, 1990).

A medium richer than LB apparently had no influence on the cell-specific hGH secretion yield, but it did affect the hormone

production per unit volume, since 2x-HKSII produced almost twice as much biomass as LB medium.

The effects of signal peptide changes on the secretion of bGH from *E.coli* were extensively studied by Klein *et al*. (1992) who failed to find any significant influence for the six alternative bacterial secretion signals investigated. In our case, on the contrary, one of the factors exerting the greatest influence on hGH secretion was the signal peptide. Simply switching from the natural hGH sequence to modified npr or to DsbA signal peptide provided statistically significant increases of 41 or 79%, respectively. The good performance of DsbA signal peptide for directing an efficient secretion and correct folding of a protein in bacterial periplasm has already been observed (Rietsch *et al*., 1996; Hannig and Makrides, 1998; Winter *et al*., 2000). Quite unexpected was the poor performance we obtained with the STII signal peptide which, together with the phoA promoter, is responsible for the highest periplasmic expression ever reported for hGH (Chang *et al*., 1987).

A statistically significant increase of hGH production (~40%) was also obtained by switching from our RRI reference strain to RB791 or W3110, but keeping all other parameters unchanged, including the temperatures of growth and activation. The latter strain not only provided the highest hGH secretion in our study, but also the highest mass fraction, reaching 20% of the total periplasmic proteins, which can greatly facilitate downstream processing of the protein of interest. *Escherichia coli* W3110 has, in fact, already been referenced as one of the most efficient strains for hGH expression and periplasmic secretion (Chang *et al*., 1987; Uchida *et al*., 1997). No report could be found on hGH production in RB791, even though this strain has proven to be able to overproduce and excrete β-lactamase or epidermal growth factor through the periplasm (Chalmers *et al*., 1990).

In conclusion, we attained a statistically significant 150% increase in hGH periplasmic expression/secretion (i.e. a cell-specific yield of 3.9 μg/ml/A_{600}) in an *E.coli* system based on the λP_L promoter after optimization of the period, stage and temperature of activation and the type of cultivation medium for four different signal peptides and six bacterial strains. Following the example of Yin *et al*. (2003), who evaluated three different promoters and three host strains for the bacterial expression of a collagen-like polymeric biomaterial in shake flask cultures, and Choi and Lee (Choi and Lee, 1997), who studied temperature, induction stage, expression time, IPTG concentration and host type, for bGH, we believe it is highly recommended that analogous optimizations always be carried out each time that a heterologous protein is expressed in a given host cell for use in scale-up production.

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