

Using L-arabinose for production of human growth hormone in *Escherichia coli*, studying the processing of gIII::hGH precursor

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Abstract

Periplasmic expression of human growth hormone (hGH) in an arabinose-regulated *Escherichia coli* system was studied, using two forms of gIII signal sequence differing from each other at position 17 (carrying either His¹⁷ or Arg¹⁷ at position -2 in the hGH precursor). The expression of hGH by the two recombinant plasmids was studied in the Top10 strain of *E. coli*. Results obtained from the expression analysis showed that the hGH expression in both of the recombinant bacteria are tightly regulated with arabinose. An optimal expression was found to occur in the medium containing 1.33 mM L-arabinose at 37°C. However, periplasmic expression of hGH occurs when the native signal sequence (His¹⁷-gIII) is applied. In the case of the bacteria carrying plasmid with the Arg¹⁷-gIII signal peptide, although the expression level was high, no mature protein could be detected under conditions tested. These data suggest for a probable effect of the -2 position in processing of the preprotein (gIII::hGH). Optimized growth and inducing conditions of the selected clone were investigated and application of a suitable signal peptide cleavage site for more efficient periplasmic production of hGH is discussed. The two recombinant plasmids presented in this work, have provided tools to study an aspect of amino acid sequence in the cleavage region on the processing and secretion of hGH.

Keywords: Recombinant human Growth Hormone (rhGH), Arabinose, Periplasmic expression, Signal peptide cleavage site

INTRODUCTION

Human growth hormone (hGH) is a small, single-chain peptide of 191 residues, produced and secreted by the anterior pituitary gland. This protein is responsible for effects in metabolism of proteins, carbohydrates and lipids as well as in growth, development and immunity (Jorgensen, 1987). Therefore, it is administered as a pharmaceutical protein in the treatment of variety of ailments and to large number of patients including hypopituitary dwarfism (Hindmarsh and Brook, 1987). The use of pituitary-derived hGH was prohibited when its association with Creutzfeldt-Jakob disease was proved (Brown *et al.*, 2000; Koch *et al.*, 1985 and Thadani *et al.*, 1988). Recombinant DNA technology has facilitated a safe and abundant production of rhGH in various heterologous systems, without risk of the transfer of human pathogens, eliminating the requirement for pituitary-derived preparations (Becker and Hsiung, 1986; Ghorpade and Garg, 1993; Gray *et al.*, 1985). Mammalian cells seem to be the most suitable host for the biosynthesis of rhGH (Catzel *et al.*, 2003). However, due to the difficulty of establishing and maintaining stable recombinant mammalian cells as well as low yields, they have not been considered as an attractive system for the production of hGH. There has been also report on the periplasmic expression of hGH in yeast using artificial signal peptide (Kohara *et al.*, 1994). Use of *E. coli* for the production of hGH is documented in several reports, including the productions in *E. coli* culture media (Hsiung *et al.*, 1989), in cytoplasm, as inclusion bodies (Mukhija *et al.*, 1995 and Schoner *et al.*, 1992) and in periplasmic space (Chang *et al.*, 1987 and Ghorpade

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and Garg, 1993). Both Met-hGH and hGH which are produced respectively in cytoplasmic and periplasmic spaces of *E. coli* are available worldwide for clinical use. Both forms have therapeutic activities that are equivalent to the pituitary-derived material (Hsiung *et al.*, 1989). At present several preparations of rhGH are available such as Saizen and Serostim (Serono Laboratories, USA), Genptonorm (Pharmacia, USA), Nutropin (Genetech USA), Humatrope (Lilly, USA) and Norditropin (Novo Nordisk, USA).

Similar to other mature secretion eukaryotic proteins, growth hormone naturally lacks formylmethionine at its N-terminal. When these proteins are produced in the cytoplasm of bacteria, they usually maintain the formylmethionine at their N-terminals (Koch *et al.*, 1985). The N-terminal methionine remains unprocessed in natural proteins containing arginine, asparagine, glutamine, isoleucine, leucine, lysine or methionine as a second amino acid in the mature protein named as penultimate residue (Vassileva *et al.*, 1999). In contrast, the initial methionine is removed efficiently when the penultimate residue is alanine, glycine or threonine and valine. In the Met-hGH, however the second amino acid is phenylalanine which has not been shown to be effective on the formylmethionine removal. Existence of anti-hGH antibody in the patients who had been treated with rMet-hGH was proven by other researchers (Massa *et al.*, 1993 and Ahangari *et al.*, 2004). Therefore it was suggested that the formylmethionine in the N-terminal of recombinant hGH probably causes the development of antibody in patients treated with rhGH. A solution for the removal of the N-terminal formylmethionine of the mature protein is to transfer the protein into the periplasmic region of the bacterial host, using a suitable signal peptide. The transfer of protein to the periplasmic region has other numerous advantages, including simplification of the downstream stages of the protein production, separation from cytoplasmic proteins, especially proteases and the concentration of recombinant proteins in an oxidizing medium, which is suitable for the formation of disulfide bonds and the refolding of the protein and finally for an active biological structure (Chang *et al.*, 1987; Goldstein *et al.*, 1990; Gray *et al.*, 1985; Kipriyanov *et al.*, 1997 and Schoner *et al.*, 1992).

Previously, we reported periplasmic expression of hGH in two IPTG/lactose inducible *E. coli* expression systems equipped with pelB signal peptide (Kiany *et al.*, 2003). Such IPTG inducible systems have provided powerful tools in basic researches. However they are not desirable in large-scale production of therapeutic proteins (Gupta *et al.*, 1999 and Makrides 1996).

Use of thermal induction is a safe alternative for the mass production of recombinant proteins (Simatake and Rosenberg, 1981). However, the temperature-inducible expression of recombinant gene products also creates problems, such as the formation of insoluble inclusion bodies at higher incubation temperatures in addition to the induction of several proteases in *E. coli* (Tabandeh *et al.*, 2004). Another option is the use of natural inducers such as lactose and L-arabinose (Guzman *et al.*, 1995). Due to low cost, tight regulation and the high level of gene expression upon induction, the L-arabinose regulated systems have been used for the expression of different proteins, including; Leader peptidase (Dalbey *et al.*, 1985), M13 gene II protein (Johnston *et al.*, 1985), interferon- α (Lim *et al.*, 2000) and HIV-1 protein (Taylor *et al.*, 1992). In this paper, the construction of recombinant plasmids for the periplasmic expression of hGH under L-arabinose induction is reported.

Our main goal is to develop an *E. coli* periplasmic expression system regulated with the natural inducer L-arabinose, for an efficient production of hGH with its authentic N-terminal. With this aim a preplasmic expression plasmid equipped with gIII signal peptide was considered as a base, in which the provided restriction sites were not suitable for the insertion of hGH cDNA immediately next to the gIII signal sequence. Therefore, two approaches were considered for a proper insertion of hGH cDNA; first the introduction of a *Hind*III restriction site to the gIII signal peptide coding sequence to be used for the junction of hGH cDNA. This approach led to a conversion of the amino acid codon at position 17 in the gIII (-2 in the preprotein) from His to Arg. In the second approach a PCR-mediated technique was applied to fuse the native gIII signal peptide to hGH. Both of the recombinant plasmids constructed in this work were examined for an efficient periplasmic expression of hGH in an arabinose regulated system in *E. coli*. In addition to optimization of growth and inducing conditions of the selected clone, the presence of two alternative amino acids (Arg and His) at position -2 in the precursor, has let us to discuss the possible functions of alternative amino acids at position -2 in a signal peptide cleavage site on the processing of their corresponding precursors.

MATERIALS AND METHODS

Bacterial strains, plasmids and primers: Top-10 strain of *E. coli* (Invitrogen-USA) was used for cloning steps as well as expression analysis. Plasmids

pBR322 and pBADgIII (Invitrogen-USA), containing arabinose promoter (P_{Ara}) were used for subcloning and periplasmic expression of hGH in *E. coli*, respectively. Plasmid pZ2-1, constructed previously (Kiany *et al.*, 2003), was used as a template for the amplification of hGH-cDNA.

Media, enzymes and chemicals: Bacteria were grown in Luria-Bertani (LB) [10 g/l Bacto-tryptone, 5 g/l Bacto yeast extract, and 10 g/l NaCl, pH 7.0 with NaOH, purchased from Merk-Germany]. Ampicillin (100 μ g/ml) was added in the culture media when required to maintain selective pressure. Enzymes *Hind*III, *Eco*RI, *Bam*HI, *Taq* DNA polymerase and T4 DNA ligase were all purchased from the Roche-Germany. Polyclonal rabbit antiserum raised against hGH was prepared kindly by Dr. Daliri in NIGEB-Iran. Immuno-reactive material was detected using horse-radish peroxidase-conjugated goat anti-rabbit antibody (Tebsan-Iran).

DNA manipulations: DNA manipulations, such as plasmid DNA isolation, DNA digestion and subclonings were performed according to standard methods (Sambrook and Russell, 2001). Commercially prepared columns (Roche-Germany) were applied for the purification of DNA from agarose gel and PCR products. Restriction analysis was employed to confirm the recombinant plasmids. The sequences of the cloned fragments were determined using AB1 373A automated sequencer (MWG-Germany).

Site-directed Mutagenesis: Two molecular approaches were applied to join the gIII signal sequence to hGH-cDNA that produced two forms of gIII signal sequence. In the first approach, a *Hind*III restriction site was introduced to the gIII signal peptide-coding fragment, which was subsequently used for the junction of signal sequence (Arg¹⁷-gIII) and hGH-cDNA. In the second approach, to join the native signal sequence (His¹⁷-gIII) to the hGH-cDNA, a PCR mediated splice-overlap- extension method (SOE-PCR) was performed as the following; first, the hGH-cDNA was amplified using primers GH-start-2 (5' TCCCAAC-TATACCACTATC3') and GH-stop-Eco (5' CCGGAATCC-TATTAGAAGCACAGCTGCC3'). In parallel, a 155bp fragment containing gIII signal peptide was amplified from pBADgIII expression vector, using primers Ara-gIII. F1 (5' TTCGGATCCTACCTGAC3') with a *Bam*HI restriction site at the 5' end and primer hGH-gIII (5' GATAGTGGTATAGTTGGGAAGCTATGGCTATAGAAC3') containing 20 nucleotides of 5' end of hGH-cDNA.

Finally, a mixture of the two PCR products was used as template for the amplification of the hybrid gIII::hGH-coding fragment.

Growth and inducing conditions: Isolated colonies obtained from frozen stocks, growing on the selective media were used for laboratory scale fermentation in 250 ml flasks (with the working volume of 50 ml). A 1/100 dilution of the overnight culture was prepared and incubated at either 30°C or 37°C until the cell density of $0.5 < OD_{600} < 1.0$ was achieved before induction by different concentrations of L-arabinose. At different time points of post-induction, the cells were harvested and total, cytoplasmic as well as periplasmic protein of the recombinant bacteria were collected, to be examined by SDS-poly acrylamide gel electrophoresis (SDS-PAGE) and western blotting.

Preparations of cytoplasmic and periplasmic proteins: Periplasmic osmotic-shock fluid from the hGH producing cells was obtained by a modified method of Libby *et al.* (1987). Briefly, at the micro-scale, 1.5 ml fermentation broth with $OD_{600} = 1$ was centrifuged at $15000 \times g$ for 5 min and pellet was collected. All the subsequent steps were carried out at 4°C. The pellets were resuspended in 15 μ l of ice cold TES buffer (0.2 M Tris-HCl, 0.5 M EDTA, 0.5 mM sucrose, pH 8.0), shaking slowly for 20 minutes. Ice-cold double-distilled water (22.5 μ l), was added to the suspended pellet and incubation continued for another 20 minutes on ice. The cells were centrifuged at $16000 \times g$ for 20 min and the pellet was stored as the cytoplasmic fraction. Trichloroacetic acid was added to the supernatant up to 12% of the final volume. The mixture was centrifuged at $16000 \times g$ for 20 min. The resultant pellet was dissolved in sample buffer and used as the periplasmic fraction. The number of cells used for the periplasmic protein analysis was maintained constant.

SDS-PAGE and Western blotting: SDS-PAGE was performed by a method described by Laemmli (1970) with slight modifications and gels were stained with comassie brilliant blue. Electroblothing of proteins onto PVDF membrane (Amersham-Pharmacia Biotech-Germany) was performed using semidry procedure, in a transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) at 86 mA for overnight. The blot was probed with a rabbit-polyclonal antiserum prepared against hGH and treated with horse-radish peroxidase-conjugated anti-rabbit antibody. The protein bands were visualized using a solution of 4-chloronaphtol with hydrogen peroxidase as its enzyme substrate.

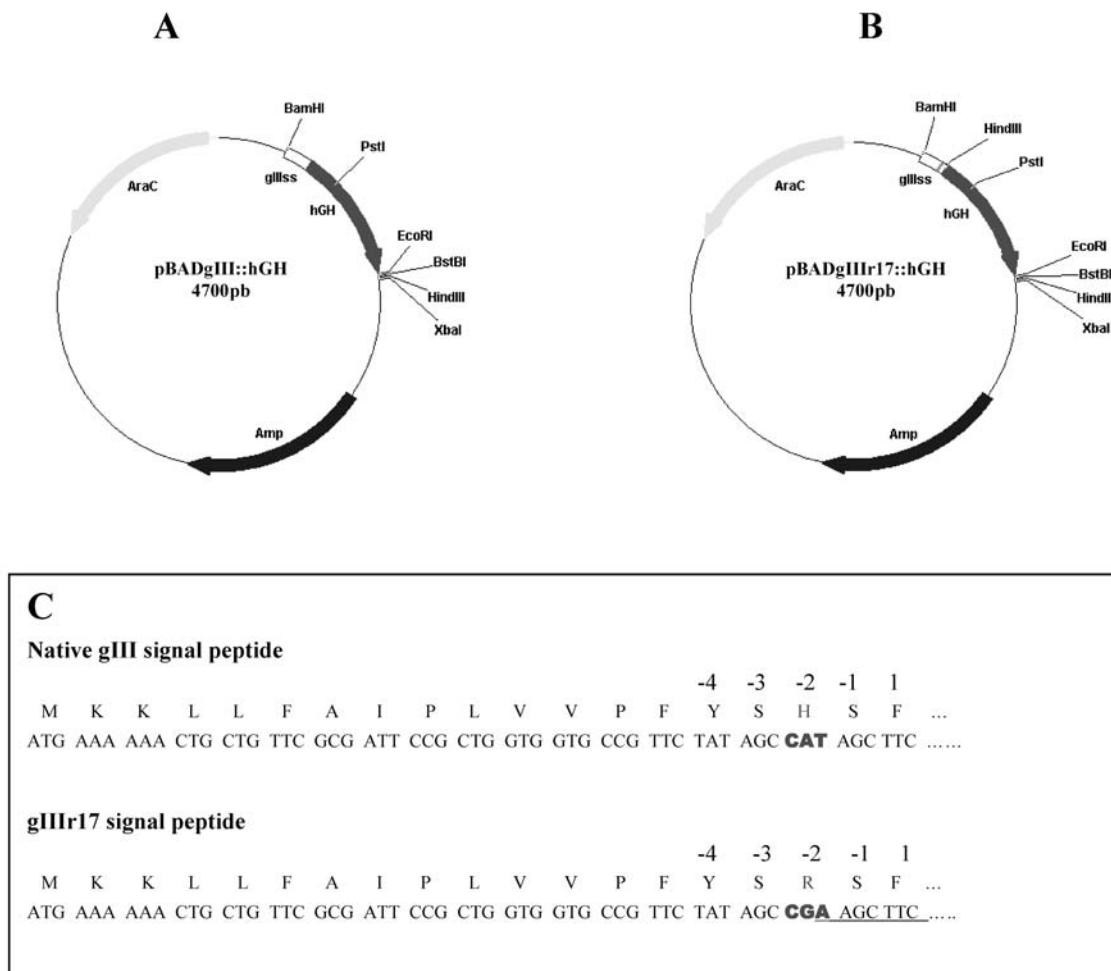


Figure 1: Schematic presentation of recombinant plasmids. Panel A: pBADgIII::hGH. Panel B: pBADgIIIr17::hGH. Panel C: the corresponding signal sequences and their junction site to hGH-cDNA of the two constructs. The *HindIII* restriction site, created by site directed mutagenesis is underlined. The His¹⁷ in the native signal peptide and Arg¹⁷ in the mutated signal peptide are shown with bold letters. The expected signal peptide cleavage site is shown by arrow head.

Expression assay of recombinant human growth hormone: The amount of expressed mature hGH was estimated by scanning the commassie brilliant blue stained SDS-PAGE of the osmotic shock-fluid, after various conditions of growth and inductions of the recombinant bacteria, by a Beckmann model R-112 densitometric gel scanner.

RESULTS

Construction of recombinant plasmids: For the construction of the pBADgIIIr17::hGH expression plasmid, we used a *HindIII* restriction site for the junction of the gIII signal and hGH-cDNA (figure 1A). The introduction of *HindIII* site converted the amino acid codon at position 17 (-2 in the preprotein) in the gIII

signal peptide from His¹⁷ to Arg¹⁷ (Fig. 1C). For the construction of second plasmid (pBADgIII::hGH), we applied SOE-PCR (see methods) to fuse the hGH-cDNA next to an intact gIII signal sequence, followed by the insertion of the *BamHI/EcoRI* restricted hybrid-fragment (gIII::hGH coding sequences) into the linearized vector with similar ends (Fig. 1B). Both of the recombinant plasmids were transferred into the Top10 strain of *E. coli*. After verification of the recombinant plasmids the recombinant bacteria were subjected for further expression analysis.

Studying the periplasmic expression of rhGH in Top10 bacteria: Following the induction with 1.33 mM arabinose, the recombinant bacteria containing pBADgIIIr17::hGH plasmid, were grown at both 30°C and 37°C separately. Total, cytoplasmic and periplas-

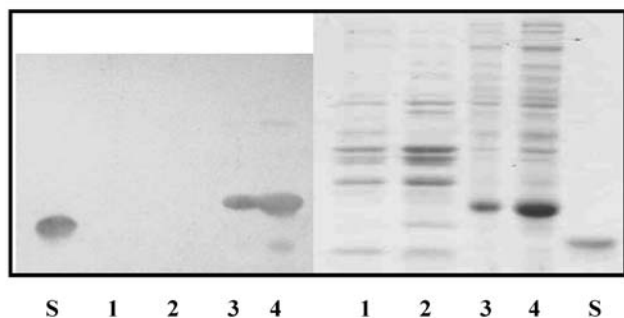


Figure 2: The proteins of the recombinant bacteria carrying the pBADgIIIr17::hGH plasmid, separated by SDS-PAGE (right) and visualized by Western blotting analysis (left) after 16h of induction with 1.33 mM L-arabinose. Lanes 1; periplasmic protein pattern at 30°C, Lanes 2; periplasmic protein pattern at 37°C. Lanes 3; cytoplasmic protein pattern at 37°C. Lanes 4; total protein pattern at 37°C. S; standard human growth hormone.

mic protein patterns of the recombinant bacteria were visualized through SDS-PAGE after 16h of induction (Fig. 2). Among the total and cytoplasmic proteins an over-expressed protein-band, detectable by anti-hGH antibody appeared (Fig. 2) which was not present in the periplasmic shock fluid. Comparing to standard hGH, the expressed protein is thought to be the unprocessed gIIIr17::hGH preprotein. Further attempts to treat the recombinant bacteria under various conditions, did not lead to the processing of the over-expressed gIIIr17::hGH fusion-protein. A similar experiment on the bacteria carrying the pBADgIII::hGH plasmid, revealed two protein-bands among their total cell proteins which were associated

exclusively with the recombinant bacteria (Fig. 3). The produced peptides were also detectable by anti-hGH antibody. The lighter band which co-migrates with standard hGH and associated with periplasmic space, was considered as processed (mature) hGH and the heavier band, collected in the cytoplasm, corresponded to the unprocessed form of hGH (gIII::hGH preprotein) (Fig. 3).

In order to predict the efficiency of the two forms of signal peptides, for their *in vivo* biological activity in an *E. coli* expression system, the cleavage sites of the two signal peptides (gIII and gIIIr17) fused to hGH-cDNA, was tested with the help of SignalP program (Nielsen *et al.*, 1999). The results obtained from the neural network-based signalP program have confirmed the experimental data obtained from the expression analysis. Accordingly, the predicted cleavage site for that of gIIIr17::hGH fusion protein is different from the expected one (Fig. 4A). Regarding the corresponding Y-score, the predicted site is not cleaved efficiently either. In the case of the native gIII signal peptide, the expected location of the cleavage site at position 19 in the preprotein was also confirmed by the program (Fig. 4B).

Assessment of the parameters involved in growth and expression: The recombinant bacteria with the native gIII signal peptide, showing potential for the periplasmic expression of rhGH, was considered for further studies, to optimize the expression of hGH in an arabinose regulated system. In this regard, a number of key parameters in expression efficiency, such as inducer concentration, growth temperature, cellular

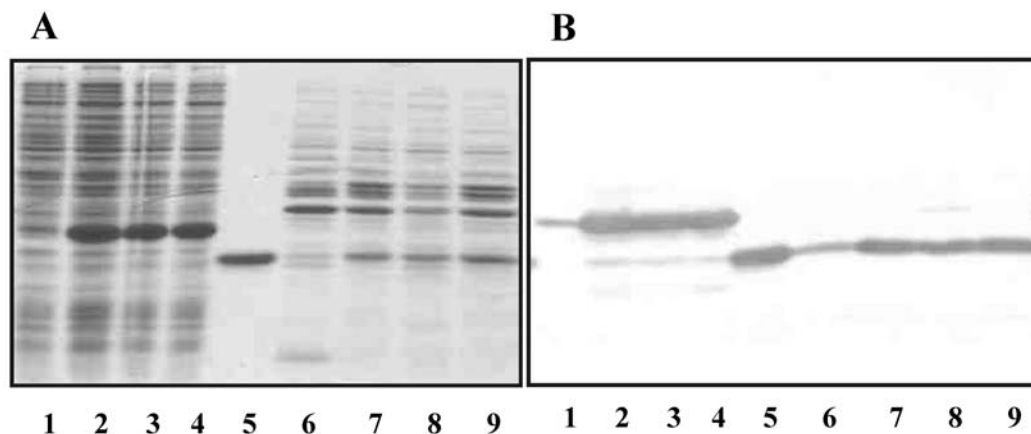


Figure 3: The Total protein patterns (lanes 1-4) and periplasmic protein patterns (lanes 6-9) of the recombinant bacteria carrying the pBADgIII::hGH plasmid after 16h of induction with various concentration of L-arabinose, separated by SDS-PAGE (Panel A) and analyzed by Western blotting experiment (panel B). Lanes 1 and 6; induced with 0.0133 mM L-arabinose. Lanes 2 and 7; induced with 0.133 mM L-arabinose. Lanes 3 and 8; induced with 1.33 mM L-arabinose. Lanes 4 and 9; induced with 13.3 mM L-arabinose. Lanes 5; standard human growth hormone.

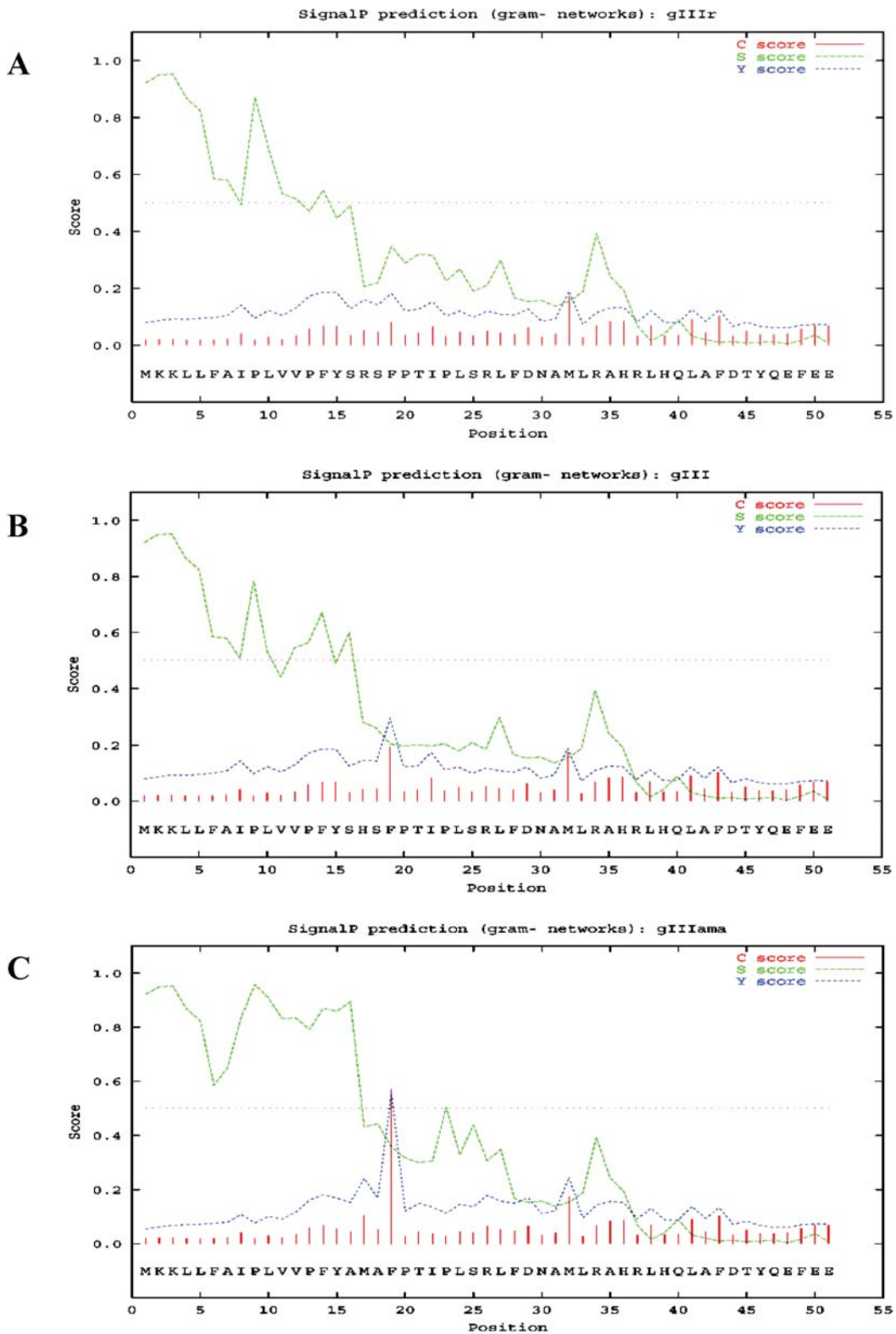


Figure 4: Prediction for the precursor of hGH with three types of GIII signal peptides with different C-terminal, performed at <http://www.cbs.dtu.dk/services/SignalP/>. C-score: output from cleavage site network. S-score: output from signal peptide networks. Y-score: combined cleavage site score. The cleavage sites are predicted with the combined peaks from C-score and Y-score on a single site. Panel A: gIIIr17 (a modified gIII signal peptide carrying Arginine at position 17), Panel B: Native gIII signal peptide (carrying histidine at position 17), Panel C: gIIIr17 (a modified gIII signal peptide carrying alanine-methionine-alanine at positions 16, 17 and 18).

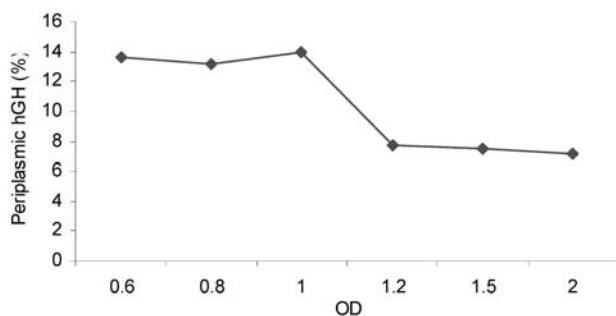


Figure 5: Effect of cell density on the periplasmic expression of hGH.

density before induction and the duration of induction were considered to be examined. After induction under various levels of each of the above mentioned factors the expression efficiency was estimated by the densitometric measurement of mature hGH related to the periplasmic proteins.

To study the effect of the host cell density, upon induction, on the periplasmic expression of rhGH, the recombinant bacteria were cultured until certain cell-densities before induction with 1.33 mM arabinose. As it is shown in figure 5 the highest level of periplasmic expression of hGH was obtained when the induction took place at the cell density of $OD_{600} = 0.5-1.0$. To evaluate the saturated concentration of inducer for hGH production using P_{Ara} , five cultures were induced in the early exponential growth phase, with 0.00133

mM, 0.0133 mM, 0.133 mM, 1.33 mM and 13.3 mM L-arabinose in shake flasks. This step was carried out at two different temperatures, 30°C and 37°C, with 4h post induction time. Based on the results, shown in figure 6, no significant difference is observed between the expression levels of hGH at the two temperatures (Fig. 6). With 1.33 mM L-arabinose, the produced mature hGH takes about 16% and 14% of the periplasmic proteins at 37°C and 30°C respectively. No basal expression of hGH was detected while 0.00133 mM or less L-arabinose was applied. However, the periplasmic accumulation of the rhGH enhanced with the increasing amount of L-arabinose (up to 1.33 mM) at the both temperatures. Above the concentration of 1.33 mM L-arabinose, there was no increase in the hGH expression level. Using the optimal concentration of L-arabinose (1.33 mM) we showed that the highest level of mature hGH expression, that is about 20% of the bacterial periplasmic proteins, is achieved after 16h of induction (Fig. 7). Longer post-induction would lead to reduction in the expression level of hGH.

DISCUSSION

Regulated expression of human growth hormone:

The L-arabinose regulated expression systems have provided the possibility of partial induction and a tight regulations on the expression of the protein (Guzman *et al.*, 1995). In the present system *araC*, which is encoded by the plasmid, act as the repressor of P_{Ara}

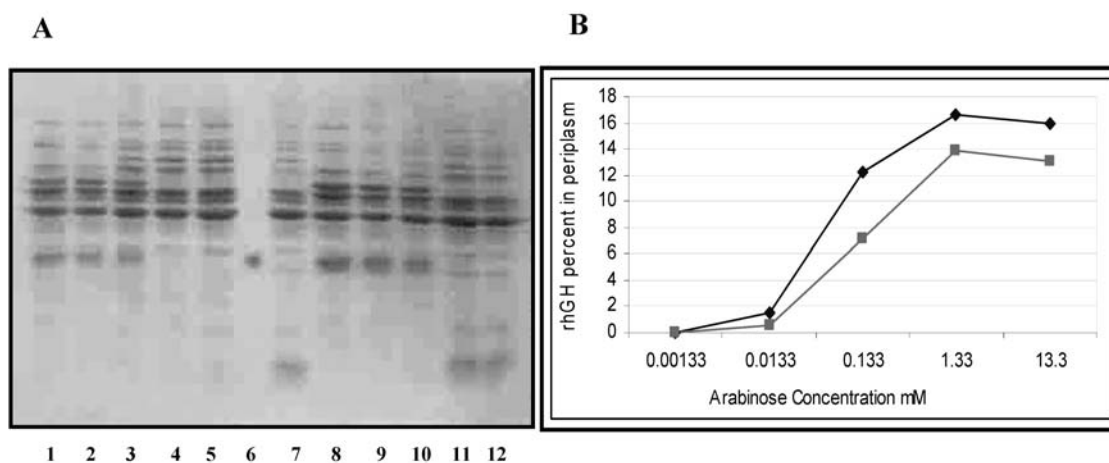


Figure 6: The periplasmic expression of rhGH with the pBADgIII::hGH containing bacteria induced with various concentrations of arabinose at either 30°C (■) or 37°C (◆). Panel A: periplasmic protein pattern of the recombinant bacteria at 30°C, Lanes 1-5: induced with 13.3 mM, 1.33 mM, 0.133 mM, 0.0133 mM, 0.00133 mM L-arabinose concentration respectively. Lane 6: standard human growth hormone (Novo). Lane 7: periplasmic protein patterns of uninduced bacteria. Lanes 8-12: the periplasmic protein patterns of the bacteria induced with 13.3 mM, 1.33 mM, 0.133 mM, 0.0133 mM, 0.00133 mM L-arabinose respectively at 37°C. Panel B: the estimation of the expressed mature hGH.

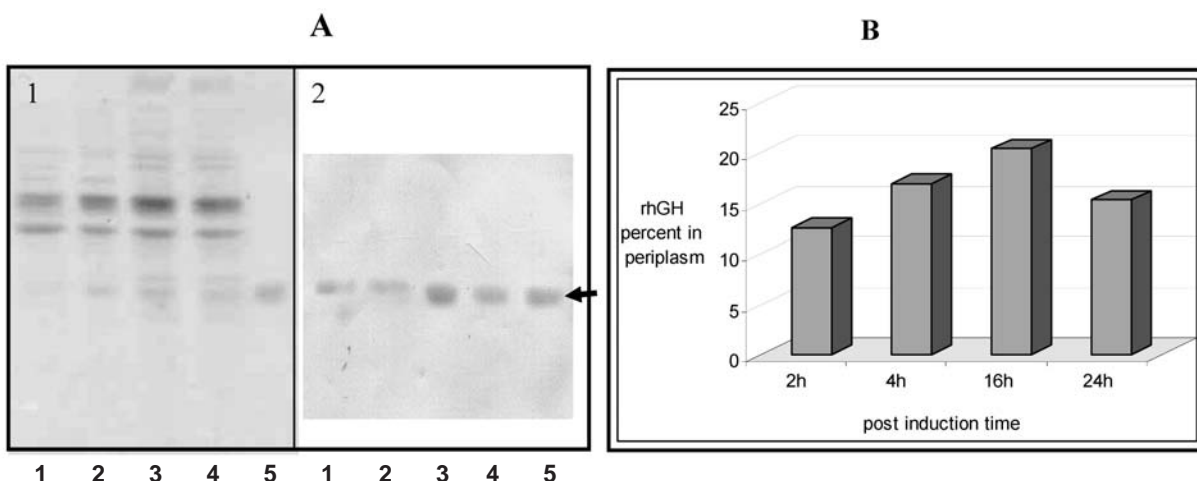


Figure 7: Time kinetics of induction of *E. coli* carrying pBADgIII::hGH plasmid. The recombinant bacteria were induced with 1.33 mM L-arabinose after growth to desirable cell concentration. At different post-induction intervals 2h, 4h, 16h and 24h, samples were taken and periplasmic fractions were prepared from a constant volume of cell cultures. Equal volumes of periplasmic fractions were electrophoresed on a 14% SDS-PAGE (respectively on lanes 1-4) (Panel A1). The electrophoresed samples were then analyzed by immunoblotting (Panel A2). The arrow indicate the mature hGH. Stabdard hGH is shown in lane 5. Estimation of the periplasmic content of hGH at different post induction intervals (Panel B).

and therefore the required amount of L-arabinose for the derepression of *araC* is theoretically proportional to the cell mass (Lim *et al.*, 2000). The curve-plateau for the periplasmic hGH in this system forms at the concentrations more than 1.33 mM of arabinose. Indeed, the highest periplasmic hGH expression occurred at the concentration of 1.33 mM of L-arabinose at 37°C after 16h of post-induction incubation, that was estimated about 16-20% of the periplasmic proteins (Fig. 7). The lack of expression below the concentration of 0.00133 mM of L-arabinose indicates in a tight regulation on the recombinant hGH in this system. The saturated concentration of L-arabinose, suggested in this study, for the periplasmic production of hGH, is comparable to that of other works in which minimum and maximum concentrations of L-arabinose were 0.1 and 1.33 mM, respectively (Lim *et al.*, 2000 and Siegele and Hu, 1997). The translation efficiency for the two constructs are apparently similar for the two produced preproteins, (i.e. gIII::hGH and gIIIr17::hGH), but with different processing efficiencies. Our results show that, the unprocessed gIII::hGH forms a major part of the expressed recombinant proteins in variuos concentrations of L-arabinose which accumulate in cytoplasm, possibly as inclusion body. The formation of inclusion bodies is most likely, the major cause of the processing inhibition of the protein precursor. In a similar study reported previously, two types of soluble and inclusion body of human IFN- α were detected in a recombinant bacteria regulated by

L-arabinose (Lim *et al.*, 2000). They showed that the percentage of IFN- α formed as inclusion bodies is increased with increasing of the L-arabinose concentration. Therefore it is thought that the optimization of L-arabinose concentration in combination with different parameters involved in expression efficiency may improve the processing efficiency of the gIII::hGH preprotein.

Effect of -2 position in cleavage region on the secretion efficiency: Several factors are involved in the processing efficiency of recombinant proteins, including; mRNA secondary structure in translation initiation region, translation initiation efficiency, maturation process, host strain, growth and inducing conditions as well as expression level (Le Calve *et al.*, 1996; Chang *et al.*, 1987; Ghorpade and Garg, 1993 and Goldstein *et al.*, 1990). These factors are very much influenced by the nature of the signal sequence and its combination with corresponding protein (Berges *et al.*, 1996). Therefore, the competence of the fusion protein being processed and translocated through the inner membrane is highly dependent on the amino acid sequences in the signal peptide (Denefle *et al.*, 1989). Signal peptides show a high degree of sequence variability but studies suggest that they function through common pathways (Kendall and Kaiser, 1998; Nilsson and Von Heijne, 1991). The carboxyl-terminal region in a signal peptide is often delimited from the h-region by one or two helix-breaking residues, and its C-terminal end

is characterized by (-3,-1)-rule or A-X-B model (Nilsson and Von Heijine, 1991; Karamyshev *et al.*, 1998). Karamyshev and his co-workers studied the function of positions -5, -4, -3, -2, -1 and +1 on the processing of *E. coli* alkaline phosphatase signal peptide (Karamyshev *et al.*, 1998). They showed that amino acid residues at positions -3 and -1 are highly conserved. Whereas, positions +1 and -2 were less conserved. Therefore, it was assumed that the recognition of the cleavage site by signal peptidase is less dependent on position -2 than that of -3 and -1. In addition to positions -6, -3 and -1, position +1 was shown to be relevant for the formation of a processing-component structure of the cleavage site region (Wrede *et al.*, 1998; Kuhn and Wickner 1985).

The two recombinant plasmids presented in this work, have provided tools to study an aspect of amino acid sequence in the cleavage region on the processing and secretion of hGH. Indeed the two constructs lead to different outcomes. With the native gIII signal peptide, at least part of the expressed protein can be translocated. Whereas, with the mutated gIIIr17 signal peptide, the expressed protein is accumulated almost completely as a precursor in the cytoplasm. In other words, the presence of arginine at position 17 in the gIII signal peptide severely impedes the processing and translocation of mature protein through the membrane. The difference observed in the outcomes of the two constructs suggests that the amino acid type at position -2 (position 17 in the gIII signal peptide) may perform a role in the gIII signal peptide processing. More relevant to the present study is the function of some amino acids at position -2 which was shown to be effective, in certain cases, on the cleavage by signal peptidase between positions +1 and +2 (Nilsson and Von Heijine, 1991). Accordingly, efficient processing is provided mainly by large amino acid residues (Phe, Tyr, Leu, His) at positions -2 and a decrease in processing occurs in the presence of small amino acid residues at position -2. Therefore, in proteins with Cys⁽⁺¹⁾ or Cys⁽⁻²⁾ or Pro⁽⁻²⁾ less processing was observed. Data collected from the distribution of residues in the cleavage region of 151 proteins from gram negative bacteria, showed that residues such as Arg, Cys, Glu, Gly and Pro do not occur at positions -2 in the tested signal peptides (Karamyshev *et al.*, 1998). No experimental data on the function of Arg at position -2 have been reported so far. Arginine is a positive charged amino acid (similar to histidine) and assumed to be the ineffective on the cleavage of the gIII signal peptide. The results obtained in the present work, showed that the presence of arginine at position

-2 inhibits the cleavage of the signal peptide. Whereas the presence of histidine at the same position leads to a proper but incompleting processing. Although the position -2 is not conserved among the signal peptides in gram negative bacteria, but the results obtained in this study suggests a probable key role for the -2 position in the secretion of the corresponding protein. Therefore further analysis are required to examine the function of different amino acids at position -2.

Based on the results obtained in this study and with the aid of the neural network based signal peptide program (Nielsen *et al.*, 1999), we predicted the processing efficiency of a modified gIII-hGH preprotein, in which positions -3, -2 and -1 in the cleavage site were converted from Ser-His-Ser to Ala-Met-Ala (Fig. 4C). Accordingly, the gIII-AMA signal peptide, compared to gIII and gIIIr17 in combinations with hGH, is expected to be processed more efficiently at the proper cleavage site. Next step to this work is to examine the efficiency of the newly designed signal peptide (gIII-AMA) for the periplasmic expression of hGH in *E. coli* experimentally.

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