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Efficient processing and export of human growth hormone by heat labile enterotoxin chain B signal sequence

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The heat-labile enterotoxin chain B (LTB) signal sequence was used for the processing and export of human growth hormone (hGH). The protein was completely processed and exported across the cell membrane to accumulate in the periplasmic space in *Escherichia coli*. The human growth hormone cDNA was cloned as a PCR amplified fragment under the control of tac promoter and translationally fused to the LTB signal sequence. The rate of processing of hGH under the control of the LTB signal sequence was equal to or more than the rate of induction of expression, indicating efficient processing. The receptor binding activity of the processed periplasmic protein was established in a radio receptor assay.

Signal sequence; Processing; Human growth hormone; Export

1. INTRODUCTION

Variation of parameters involved in expression of recombinant proteins is a phenomenon commonly observed in designing strategies for genetically engineered proteins. Different permutations and combinations of vectors, promoters, signal sequences and regulatory signals have been tried for improving the quality and quantity of recombinant proteins. The human growth hormone being a protein of immense biomedical importance, a number of strategies have been employed for its expression. hGH being a non-glycosylated protein, prokaryotic expression systems have been preferred to mammalian or yeast expression systems. It has been well documented that recombinant proteins expressed in *E. coli* can remain soluble in the cytoplasm [1], become sequestered into inclusion bodies [2] or be excreted across the cytoplasmic membrane to accumulate in the periplasmic space [3,4]. Expression of hGH in *E. coli* under the control of trp promoter formed inclusion bodies that led to difficulties in purification and folding of the recombinant protein [5]. True secretion in *E. coli* is rare. A variety of signal peptides from *E. coli* have been tried to achieve export of soluble hGH to the periplasm [6–8]. The strategy to use the signal peptide to make the protein enter the secretary pathway has been successful; however, it also has encountered some problems. Most commonly, the processing of the precursor polypeptide is not always efficient and leads to losses of protein in the form of cytoplasmic retention of unprocessed protein. This has been observed with ompA signal peptide for hGH, human interleukin-1β (human IL-1β) and alkaline phosphatase signal sequence for IL-1β in *E. coli* [3,9]. Alternate hosts like *Pseudomanas*, *Bacillus subtilis* have also been tried and even these systems do have similar problems associated with it. Native signal sequences employed for export were not able to direct the protein in the export pathway. This was observed when native human tissue plasminogen activator and ovine pre-growth hormone were expressed in *E. coli* and pre-hGH was expressed in *Pseudomanas* [10–12]. The neutral protease gene signal gave partial processing of hGH in *Bacillus* [13,14]. The quest for the ideal combination of the host, the promoter and the signal sequence, thus, continues.

In the present study, we report the efficient processing of human growth hormone by the heat-labile enterotoxin chain B signal sequence. Upon export across the cell membrane the mature protein accumulates in the periplasmic space in *E. coli*.

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Abbreviations: hGH, human growth hormone; LTB, heat-labile enterotoxin chain B; IPTG, isopropyl-β-D-thio-galactopyranoside, pGGhGH-E, plasmid harboring hGH gene under the tac promoter and LTB signal sequence.

2. MATERIALS AND METHODS

2.1. *Enzymes and chemicals*

DNA restriction and modification enzymes were purchased from New England BioLabs, USA. Isopropyl-β-D-thio-galactopyranoside (IPTG) and ampicillin were from Boehringer Mannheim, Germany and Sigma Chemicals, USA respectively. Bacto-tryptone, Bacto-yeast extract and Bacto-agar were from Difco Laboratories, Detroit, USA.

2.2. *Bacterial strains, plasmids and media*

E. coli HB101 cells were used as hosts for all transformations Plasmid analysis and transformation were carried out as described

[15]. Plasmid pMMB68 was a gift from Dr. Jan Holmgren. Commercially available plasmid pUC18 was used for cloning.

2.3. *Construction of plasmid pGGhGH-E*

Convenient cloning sites were created by PCR amplification using primers harboring *Sac*I and *Hin*dIII sites at the 5′ and the 3′ ends respectively. The N-terminal methionine present in the template plasmid ptrpcGH [5] was deleted. The amplification product (560 bp) was digested with *Sac*I and *Hin*dIII enzymes and cloned in pUC18 to yield plasmid pUCcGH(+1) which served as the source of the insert for further construction. A +1 frameshift was introduced while re-creating the *Sac*I cloning site, which was corrected in plasmid pGGhGH-E using synthetic oligonucleotides. The cGH fragment excised from plasmid pUCcGH(+1) with *Sac*I and *Hin*dIII enzymes was further digested with *Hin*fI and the *Hin*fI–*Hin*dIII fragment was used in a triplet cloning strategy alongwith synthetic oligonucleotides to construct plasmid pGGhGH-E. The detailed cloning strategy is as given in Fig. 1. The first amino acid of LTB was retained at the junction of the signal sequence and mature protein. The +1 site is thus occupied by an Ala residue.

2.4. *Induction of hGH expression and preparation of periplasmic fractions*

E. coli cells harboring the plasmid pGGhGH-E were grown for 10–12 h in Luria Bertani medium (LB) with 50 μg/ml of ampicillin. Freshly subcultured 0.5 A_{600}/ml unit cells were induced with 1 mM IPTG for different time periods (0–48 h). Periplasmic fractions were prepared by the osmotic shock treatment [16].

2.5. *Immunoblot analysis of hGH expression*

The periplasmic and the cytoplasmic fractions or the total cell extracts were electrophoresed on 15% SDS-PAGE and the separated proteins were electrotransferred onto a nitrocellulose membrane at 30 mA for 10 h. Non-specific sites were saturated using 1% non fat milk powder in 50 mM PBS (pH 7.2) for 1 h. The membrane was then incubated with goat anti-hGH polyclonal antiserum followed by an incubation with anti-goat IgG HRPO conjugate. The membrane was washed thoroughly with PBS containing 0.05% Tween-20 between successive incubations. Immunoreactive protein bands were developed using 4-chloro-1-naphthol as substrate.

2.6. *Receptor binding activity of recombinant hGH*

Crude plasma membrane fraction was prepared using homogenized rat liver tissue by a modified protocol as described [17]. [^{125}I]hGH was prepared as described by Fraker and Speek [18]. The receptor binding acitvity of hGH was assayed in a radio receptor assay (RRA) using periplasmic fractions of the cells using appropriate modifications of the RRA protocol described earlier [17]

3. RESULTS AND DISCUSSION

3.1. *Analysis of the expression of hGH by pGGhGH-E*

The plasmid pGGhGH-E, obtained as described in Fig. 1, contains the tac promoter along with the heat-labile enterotoxin chain B signal sequences functionally linked to the cDNA of the human growth hormone. *E. coli* cells harboring plasmid pGGhGH-E were induced for expression with IPTG (Fig. 2). A band comigrating with the standard hGH preparation was observed in the periplasmic fraction of the induced cells that could be immunoblotted with anti-hGH goat polyclonal sera (lane 5). A stringent control on inducibility was observed as no leakage of expression is observed in the periplasm of uninduced cells (lanes 4 and 6). The total cell extract of the induced cells also showed a single band corresponding to that of mature hGH and no band of the unprocessed molecule was detected (lane 7). As the polyclonal antiserum raised against hGH can detect the unprocessed molecule (unpublished data). Unlike the concept of secretory load perceived by several other researchers, our results clearly indicate that the processing of the polypeptide resulting in the cleavage of the signal peptide followed by the export of the mature protein in the periplasmic space is a very efficient process.

In *E. coli*, the outer membrane protein A (ompA) signal peptide used for hGH showed unprocessed protein [3]. The human IL-1β, when expressed as a fusion with the alkaline phosphatase (phoA) signal sequence

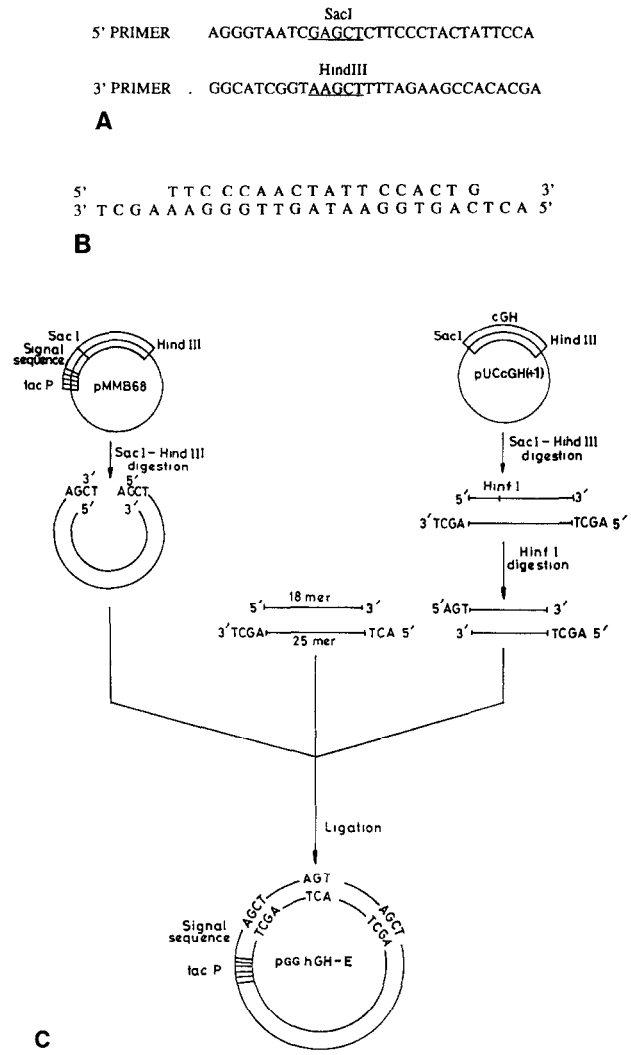

Fig. 1. Cloning strategy for construction of plasmid pGGhGH-E. (A) The synthetic oligonucleotide primers harboring the appropriate sites at the 5′ and 3′ ends used for the PCR amplification of the synthetic cDNA of hGH (B) Sequence of oligonucleotides used for triplet cloning (C) Construction strategy for plasmid pGGhGH-E. The 560 bp hGH fragment having *Sac*I at the 5′ end and *Hin*dIII at the 3′ end generated by the PCR amplification of plasmid ptrpcGH was used as a source of insert. All fragments were purified by electroelution as described [15]. Hatched box indicates tac promoter and dotted lines indicate LTB signal sequence.

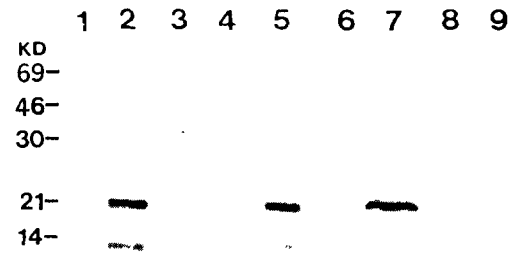

Fig. 2. Western immunoblot analysis of *E. coli* pGGhGH-E. Periplasmic fractions, cytoplasmic fractions and total cell extracts of *E. coli* pGGhGH-E cells grown in the presence or absence of IPTG were electrophoresed followed by an elecrotransfer onto a nitrocellulose membrane at 30 mA for 10 h. The membrane was processed for the detection of hGH using a goat anti-hGH polyclonal antiserum and anti-goat HRPO conjugate as described in text. Lanes 3–5 represent periplasmic fractions of *E. coli* HB101 cells, *E. coli* pGGhGH-E cells uninduced and induced respectively. Lanes 6 and 7 correspond to the total cell extracts of uninduced and induced *E. coli* pGGhGH-E cells. Lanes 8 and 9 correspond to cytoplasmic fractions of uninduced and induced *E. coli* pGGhGH-E cells. Standard hGH (500 ng) was used as control (lane 2). Lane 1 represents molecular weight markers. The arrows indicate the 22 kDa and 14 kDa hGH bands

and ompA signal sequence, gave only 20% and 50% of processed protein respectively [9]. The use of neutral protease gene signal sequence also showed partial processing of hGH in *Bacillus* [14]. When the neutral protease gene prepropeptide was used for the secretion of hGH in *Bacillus*, the unprocessed protein along with the 48 amino acid prepropeptide was excreted in the early hours of secretion phase due to inefficient processing and aberrant secretion [13]. In our system, however, the rate of the processing is equivalent or more than the rate of induction of expression, since, only the mature form of growth hormone can be detected in the cell at any given time. The cytoplasm of the cells is devoid of any hGH suggesting that the protein detected in the total cell extract originated from the periplasm alone. These observations are, however, consistent with the earlier observations; experiments carried out to study bacterial export necessitated the construction of special strains where the leader peptidase was made limiting in order to elucidate the temporal order of events [19]. Under the normal circumstances the sheer efficiency of the export apparatus made such determinations technically impossible. In addition to the 22 kDa band corresponding to the native and intact hGH, another immunoreactive band migrating at 14 kDa can also be observed in the periplasmic fractions as well as the total cell extracts of cells induced with IPTG (Fig. 2, lanes 5,7). It has been well documented that, when hGH is expressed as a periplasmic protein a finite fraction of the product is produced as a cleaved product. *E. coli* cells transformed with plasmids engineered to express hGH in the periplasm, produce a proteolytically cleaved form of hGH [20]. A certain proportion of the molecules is cleaved to a two-chain form by an enzyme which may be located in the cell membrane. The behavior of the two-chain form in reducing SDS-PAGE gives a 22 kDa and an additional 14 kDa protein. The two-chain form can displace [^{125}I]hGH from a rabbit liver receptor preparation as well as intact rhGH [20]. The extracellular hGH expressed in *Bacillus subtilis* was also expressed as a two-chain form [21,13].

3.3. *Effect of inducer concentration and time kinetics of hGH expression*

The effect of inducer concentration on hGH expression was studied using different concentrations of IPTG at a constant induction time. The results of this experiment are shown in Fig. 3. It was observed that, even the lowest concentration of IPTG used, i.e. 0.01 mM, could induce the expression of hGH. Increasing concentrations of the inducer until 0.5 mM led to an increase in the expression levels; however, any further increase in the IPTG concentration did not lead to any detectable increase in the expression levels. In order to analyze the time course of induction at a given inducer concentration, kinetic studies were performed. The post-induction growth curve of *E. coli* harboring the plasmid pGGhGH-E showed that the cells were in active log phase of growth 12 h post-induction, after which they entered the stationary phase that lasted until 24 h and was followed by the death phase of the growth curve (data not shown). The results of the time kinetics of hGH expression are given in Fig. 4. The number of cells used for extracting the periplasmic fraction was maintained constant. Immunoblot analysis of the periplasmic fractions showed that the induction levels seem to increase until 12 h post-induction beyond which they plateaued. Processed hGH was detected in samples withdrawn immediately on addition of the inducer (Fig. 4, lane ot). As the cells do not have any detectable hGH

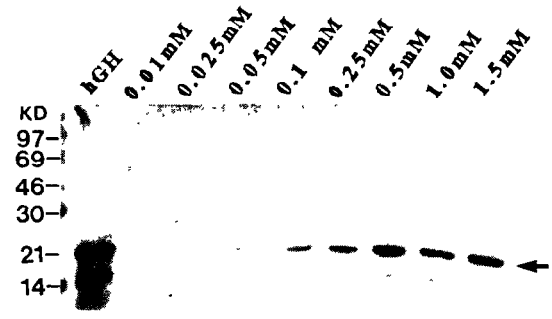

Fig. 3. Effect of inducer concentration on the expression of hGH : 0.5 A600/ml unit *E. coli* pGGhGH-E cells were induced for 6 h with 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, 1.0 and 1.5 mM IPTG. Periplasmic fractions of these samples were immunoblotted by using a goat anti-hGH polyclonal antiserum. Standard hGH (500 ng) is used as control. MW corresponds to molecular weight markers. The arrow corresponds to mature hGH.

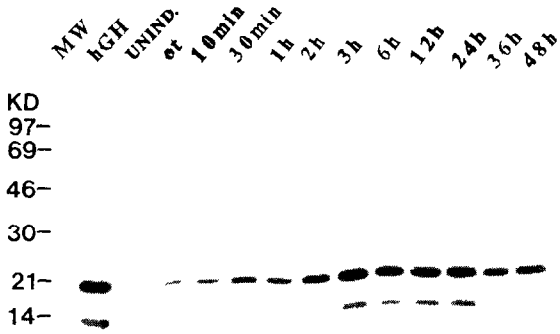

Fig. 4. Time kinetics of induction of *E coli* pGGhGH-E. *E coli* pGGhGH-E cells were induced with 1 mM IPTG. At different post-induction intervals, samples were taken and periplasmic fractions were prepared from 5.0×10^8 cells each. Equal volumes of periplasmic fractions were electrophoresed on a 15% SDS-PAGE The electrophoresed samples were then analyzed by immunoblotting. The arrows indicate the mature hGH. hGH is standard preparation (500 ng). MW indicates molecular weight markers

prior to induction (lane UNIND.), the appearance of the protein immediately on induction can be attributed to the sample processing time amounting to a few minutes. Thus, it can be inferred that the onset of induction is very efficient. Fig. 5 shows the analysis of the total cell extracts as a time course of hGH expression. The amount of total hGH detected in the culture follows a similar pattern like that of growth curve as both are a function of cell number. No residual unprocessed protein is detected in the cells indicating that the increasing protein load does not lead to inefficient processing and export in this case even after cells enter the late phases of growth.

It has been reported that the expression of some inducible recombinant proteins is improved on lowering

Fig. 5. Time course of induction of *E coli* pGGhGH-E. *E. coli* pGGhGH-E cells were induced with 1 mM IPTG. At different time points equal culture volume was withdrawn and the cell pellet was resuspended in reducing SDS-PAGE sample buffer. After boiling and centrifugation, equal volume of supernatant was analyzed on a 15% SDS-PAGE and immunoblotted as described. hGH is standard preparation (500 ng). MW indicates molecular weight markers.

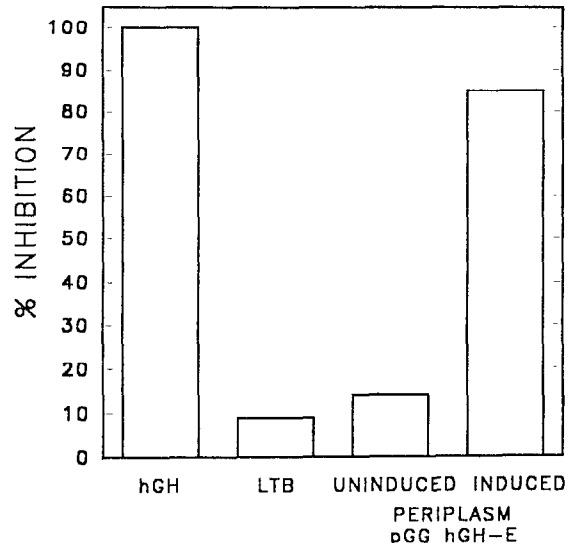

Fig. 6. Specificity of the radioreceptor assay. The plasma membrane preparation having the receptor prepared as described in the text was assayed for specificity of hGH binding. LTB was used as a nonspecific competitor. Periplasmic fractions of *E coli* HB101 cells and *E coli* pGGhGH-E cells with and without IPTG induction were assayed.

the induction temperature to 28°C. However, in our system, no significant difference in the expression levels was observed at 28°C (data not shown).

3.4. *Analysis of the receptor binding activity of recombinant hGH*

To establish the receptor binding activity of the protein exported with the LTB signal sequence, a radio receptor assay (RRA) was standardized using a rat liver membrane preparation. Results are shown in Fig. 6. The receptor assay was shown to be specific for hGH. However, in the RRA, the periplasmic fractions of the uninduced cells brought about 14% inhibition of the receptor binding as compared to the 85% inhibition observed with induced cells. This indicates small amount of hGH present in the cells prior to induction. The detection of leakage of expression only by RRA, an assay more sensitive than the immunoblot, indicates that the levels of leakage are probably minimal.

In this report, we clearly demonstrate that the use of heat-labile enterotoxin chain B signal sequence brings about complete and efficient processing and export of human growth hormone in *E. coli*. The protein accumulates in the periplasm on cleavage of the signal peptide as a soluble protein and the recombinant protein is capable of binding to the receptor.

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