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Enhanced periplasmic expression of human Granulocyte Macrophage Colony Stimulating Factor in *Escherichia coli* by the modification of the signal peptide cleavage site

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Abstarct:
Human ganulocyte macrophage colony stimulating factor (hGM-CSF) is one of the hematopoetic growth factors and regulator of hematopoesis that has been used as therapeutic agent in treatment of various ailments, such as myelodysplastic syndrome, neutrogena and chemotherapy induced myleosuppression. With the aim of the large-scale production of recombinant hGM-CSF in the periplasmic space of *E. coli* a fusion fragment coding for pelB::hGM-CSF, constructed by splice overlap extension PCR (SOE-PCR) technique, was inserted in a T7-based expression plasmid. The expression analysis was performed on the IPTG (or lactose)-induced BL21 (DE3) strain of *E. coli*, containing the recombinant plasmid. Using SDS-PAGE and densitometric assay of protein bands corresponding to the recombinant hGM-CSF, we analysed periplasmic expression of hGM-CSF after induction with either 1mM IPTG or various concentrations of lactose. Both the processing of signal peptide and the translocation of the mature hGM-CSF took place more efficiently when lactose was used as inducer, comparing to the results obtained from the IPTG induction. The optimum concentration of lactose was found to be 0.01% after 20 hours, lead to an over-expression of pelB::hGM-CSF followed by nearly complete processing and transport of the mature protein into in the periplasmic space. The mature hGM-CSF expressed in the present construct was estimated around 60% of the bacterial periplasmic proteins. Comparing to a previously-made hGM-CSF expressing plasmid (K1), which carries six extra-nucleotides, coding for methionine and alanine, in the signal peptide C-terminal, the periplasmic expression efficiency of the newly made plasmid is higher both at processing level and the translocation of mature protein into the periplasmic space. These data support the idea that amino acid context in the cleavage site region play a key-important role in the expression efficiency of secretory proteins. Moreover it was documented that a combination of the improved signal peptide cleavage site and the use of an optimized lactose concentration can lead to a highest of production level of mature hGM-CSF.
Keywords: human GM-CSF, lactose, IPTG, T7/lac over- expression system, induction, *Escherichia coli* and recombinant protein, signal peptide & processing.

Introduction:

Human GM-CSF is a 127 amino acid protein and one of the hematopoetic factors that controls and stimulate the production and activation of granulocytes and macrophages from progenitor cells (3, 6). Yet *E. coli* offers the advantage of its well-known genetics as well as the availability of numerous efficient vectors. Therefore, we choose this host for studying the expression of hGM-CSF. In a previous work, we reported the expression of recombinant hGM-CSF to the bacterial periplasmic space using K1- plasmid (figure 1), which carries six extra-nucleotides in the signal peptide C-terminal (1). In the periplasmic space, the expressed protein may find better conditions for proper folding, especially for the formation of stabilizing disulfide bonds. In present work in addition to improvement of the precursor cleavage of the previously made plasmid, we examined the application of lactose to induce the expression of rhGM-CSF.

Matherials and methods:

A PCR-mediated technique, Splice Overlap Extension-PCR (SOE-PCR), was applied to join the hGM-CSF protein and pelB signal peptide coding regions through three PCR reactions as the following**.** PCR 1 was performed for the production of the pelB coding fragment. PCR 2 was performed for the production of hGM-CSF coding fragment and finally PCR 3 was carried out For the production of the fused pelB::hGM-CSF coding fragment, mediated with the PCR1 and PCR 2 products. Plasmid DNA isolation, DNA digestion and sub-cloning steps were performed

according to standard methods (5). Commercially prepared columns (*Roche-Germany*) were also applied for the purification of plasmids and DNA fragments from agarose gel as well as PCR products. In addition to restriction analysis, PCR approach was taken to confirm the recombinant plasmids, followed by nucleotide sequence analysis (*MWG-Germany*). The comparison of the obtained sequences against the Gene-Bank was performed using Blast program. SDS-PAGE was performed by a modified method described by Lammli (2) and gels were stained with commassie brilliant blue. Electroblotting of proteins onto nitrocellulose PVDF membrane (*Roche*-Germany) was performed using either wet blotting or semi-dry blotting procedure (4).

Results & discussion:

The fused fragment coding for pelB::hGM-CSF, was inserted in the *Nde*I/*Bam*HI sites of a pET26(b+) plasmid after digestion with the same enzymes. The 5734 bp recombinant plasmid (figure1B) was transferred into the BL21 (DE3) strain of *E. coli* and the recombinant bacterium was named (H13). After verification, the H13 clone was subjected for further analysis comparing to the previously made clone containing the K1 plasmid (figure1A).

Expression analysis; Using SDS-PAGE, both total as well as periplasmic protein patterns of the isolated BL21 clone (H13) taken 4 and 20 hours after induction with either IPTG (figure 2A) or different consentrations of its natural analogue, lactose, (figure 2B) were analysed. The results, indicated in the over expression of a protein, that was comparable to the standard hGM-CSF in size. This was further confirmed by its reaction with the rabbit serum directed against hGM-CSF (figure2C). A larger protein, which is thought to be unprocessed pelB::hGMCSF precursor, exclusively associated to total as well as cytoplasmic proteins but not the periplasmic fraction of the recombinant bacteria (figures 2A & 2C).

Comparison of Clone H13 and K1 for expression and processing efficiency; The total protein patterns of the induced H13 and K1 clones were subjected for the estimation of the relative amount (in percent) of mature as well as precursors of the expressed hGM-CSF in 4 and 20 hours after induction. As it is outlined in figure 3, although the expression efficiencies of the both clones relatively similar when IPTG is used as inducer, but the processing of the signal peptide and transport of the mature protein to the periplasmic space is higher in the case of clone H13. The difference between the two clones is even more significant when lactose is used as inducer. The highest level of the mature hGM-CSF for clone K1 appears at 20 hours of induction with 0.05% lactose that is about 16% of the proteins of the K1 clone. Whereas the highest level of mature hGM-CSF for clone H13 appear at 20 hours of induction with 2% lactose that is about 25% of proteins of the H13 clone. Comparing the relative amounts of the mature(M) and precursors (P) of samples obtained after various inducing conditions of the two clones, it is thought that the processing efficiency, that is defined as the presence of the lowest amounts of unprocessed protein (pelB::hGM-CSF) in cytoplasm, occurr after 20 hours induction of clone H13 with 0.01% lactose.

Optimization of the inducing condition of clone H13; We have also estimated the relative amounts of mature hGM-CSF among both periplasmic and total proteins of the clone H13 in various inducing conditions (figure 4). These results also show that the highest periplasmic expression level in clone H13 occurs after 20 hours induction with 0.01% lactose, which is in agreement with our previous experiment, outlined in figure 3. Accordingly, the mature hGM-CSF expressed in the present construct was estimated around 60% of the bacterial periplasmic proteins.

Based on the results obtained so far, comparing to a previously-made (K1) GM-CSF expressing plasmid, which carries six extra-nucleotides, coding for methionine and alanine, in the signal peptide C-terminal, the periplasmic expression efficiency of the newly made(H13) plasmid is higher both at processing level and the translocation of mature protein into the periplasmic space.

References:

1- Borjaliloo, S., Zomorodipour, A., Yakhchali, B., and Shojai, S. *The Iran J Biotechnol*(2003). Vol 1. No 2 .101-108
2- Lammli, UK. (1970). *Nature*. Vol, 227: 680-685.
3- Lee, J. D. and Ramirez, W. F. (1992). *Biotech. Bioeng.* **39**, 635-646.
4- Libby, R., Braedt, G., Kronheyim, S., March, C. J. Urdal, D. L., Chiaverotti, T. A., Tushinski, R. J., Muchizuki, D. Y., Hopp, T.P., Cosman. D. (1987) *DNA*, Vol. 6: 221-229.
5- Sambrook J, Rucell D. (2001) Molecular Cloning: A Laboratory Manual
6- Welte K, Platler E, Lu L, Gabrilove JL, Levi E, Mertelsmann R, Moore MAA. (1985) *Proc. Natl. Acad. Sci. USA.* Vol. 82: 1526-1530.

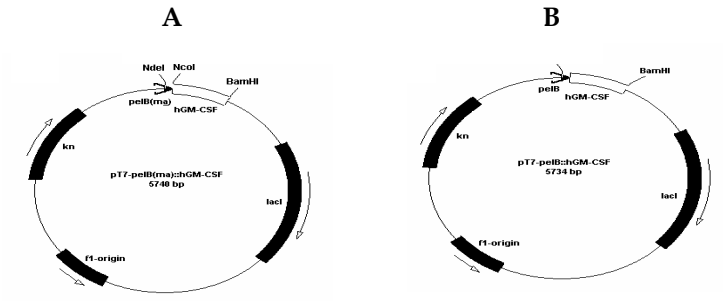

Figure 1: The physical maps of recombinant plasmids; A: The K1 plasmid with two additional amino acids codons in the cleavage site between signal sequence and the hGM-CSF cDNA, B: with native pelB signal sequence fused to the hGM-CSF cDNA produced by SOE-PCR

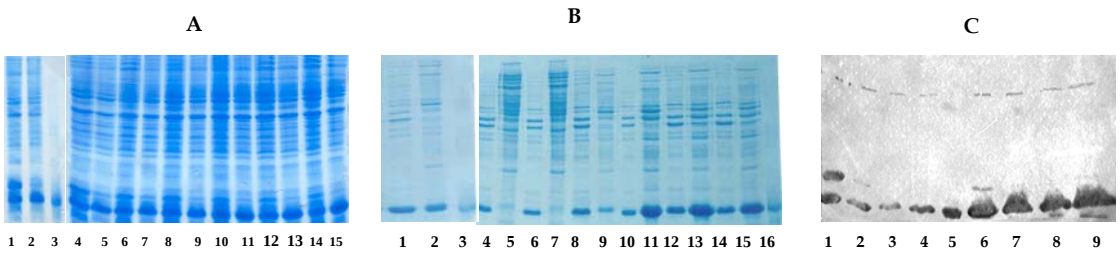

Figure 2: Expression analysis of the clone H13 after 4 and 20 hours of induction. Panel A: SDS-PAGE analysis of the total proteins. Panel B: SDS-PAGE analysis of periplasmic proteins clone. Panel C: immunoblotting experiment of the total proteins. Panel A & B: Lanes 1 & 2, after 4 and 20 hours of induction with IPTG. Lane 3, standard hGM-CSF. Lanes 4 & 5: after 4 and 20 hours of induction with 2% actose. Lanes 6 & 7: after 4 and 20 of induction with 1% lactose. Lanes 8 & 9: after 4 and 20 hours of induction with 0.5% lactose. Lanes 10 & 11, after 4 and 20 of induction with 0.1% lactose. Lanes 12 &13: after 4 and 20 hours of induction with 0.05% lactose. Lanes 14 and 15: after 4 and 20 hours of induction with 0.01% lactose. Lane 16 standard hGM-CSF.**Panel C:** Lanes 1-4: after 4 hours of induction. Lanes 6-9: after 20 hours of induction. Lanes 1 & 6: Induced with 1mM IPTG. Lanes 2 &7: Induced with 0.1% lactose. Lanes 3 & 8 : Induced with 0.05% lactose. Lanes 4 & 9: Induced with 0.01% lactose. Lane 5: Standard HGM-CSF.

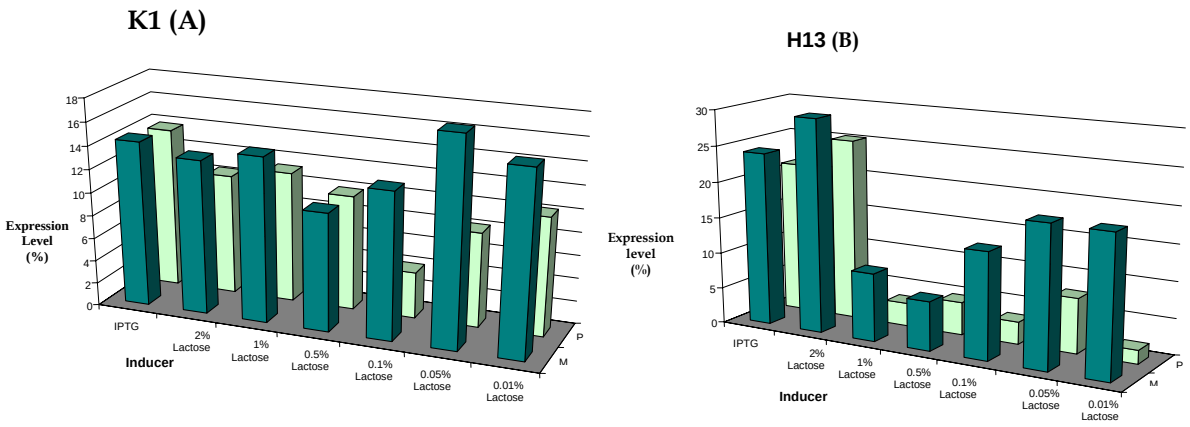

Figure 3: Comparison of the processing efficiency of the expressed rhGM-CSF precursor between the two recombinant bacteria K1 (Panel A) and H13 (Panel B). Estimation (in percent) of the mature as well as precursor of rhGM-CSF relative to total recombinant bacterial proteins, 20 hours after induction with either IPTG or various concentrations of lactose (0.01-2%).

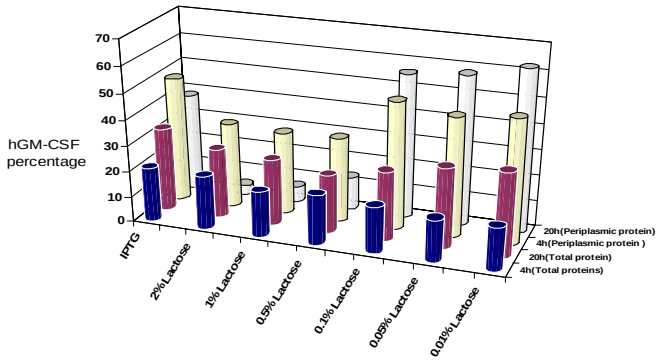

Figure 4: Estimation (in percent) of the mature rhGM-CSF relative to total and periplasmic proteins 4 and 20 hours after induction with either IPTG or various concentrations of lactose (0.01-2%), produced by the recombinant bacteria (H13).