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Cellular response to accumulation of recombinant proteins in the *E. coli* inner membrane: Implications for proteolysis and productivity of the secretory expression system

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Abstract

Several literature reports have indicated adverse effects on host cell growth and physiology due to secretory expression of recombinant proteins. In this work, extensive proteolysis of recombinant streptokinase was observed in the secretory expression system, along with severe growth impairment and reduced productivity as compared to intracellular expression in *Escherichia coli*. These phenomena correlated well with the accumulation of the secretory recombinant protein in the inner membrane and a corresponding up-regulation of an inner-membrane protease (probably ClpX). This protease was found to be activated only in the post-induction phase of the secretory expression system. A reduction in the cultivation temperature led to a significant decrease in the accumulation level of recombinant streptokinase in the inner membrane and a concomitant decrease in the inner-membrane protease activity. This resulted in a considerable enhancement in the overall productivity of the secretory expression system. The results demonstrate that the productivity of the secretory expression system is controlled by the accumulation level of the recombinant protein in the inner membrane. Beyond a particular accumulation level the cellular response triggered in the form impaired growth and enhanced proteolysis significantly affects the productivity of the system. In order to reduce the accumulation in the inner membrane, the synthesis rate needs to be tuned to the protein-specific translocation efficiency of the cell.

Keywords: *Escherichia coli*; Cellular stress response; Secretory expression; Inner-membrane protease; OmpA signal peptide; Co-translocational proteolysis

1. Introduction

Escherichia coli has been widely used as an expression host for the production of high-value recombinant proteins [1,2]. Recombinant proteins can be synthesized in *E. coli* and then targeted to different compartments [3]. Secretory production of recombinant proteins in *E. coli* has been beneficial for several reasons [4]. These include the proper formation of disulphide bonds due to oxidative environment in the periplasm [5–7]; correct processing of the N-terminal amino acid [8]; reduced protease activities in the periplasm compared to the cytoplasm [9,10]; preventing the formation of insoluble, biologically-inactive products [11]; and ease of purification as the content and the number of protein impurities is much lower in the periplasm than the cytoplasm [12].

Even though secretory expression of recombinant proteins has been beneficial, hyper-secretion of recombinant proteins has adverse effects on the host cells and on the stability of the recombinant proteins produced. These effects include impaired growth or complete cell death after the induction of the recombinant protein [13–15]; plasmid instability or plasmid curing [16]; and proteolytic degradation of the recombinant protein [17]. Thus, the overall productivity of the system is considerably reduced due to these adverse cellular responses.

Like any other biological process, protein secretion is a complex process involving coordinated function of several individual processes (such as synthesis rates of the pre-protein, folding characteristics of the recombinant protein, processing of pre-protein, translocation, etc.). Understanding of the protein secretion mechanisms in *E. coli* has led to the development of various strategies for enhancing secretory production of recombinant proteins [8]. The strategies range from selection of the type of secretion pathway to detailed manipulation of

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every possible step involved in protein secretion. These include transcription rates [18,19]; translation rates [20]; interaction with cytoplasmic secretion modulators [21]; altered translocation efficiency brought about by signal peptide engineering [22,23]; enhanced translocation capacity brought about by increasing the secretion channels [24,25]; and post-translocation folding [8].

Most of the above mentioned biological strategies have generally examined modulation of one particular step for enhancing secretion efficiencies. However, manipulating the elements of a complex process, one at a time, may not be always beneficial for increasing the overall productivity of the system. This is because each of these individual processes is regulated by several other factors. Therefore, implementation of several of the biological strategies mentioned above has resulted in different levels of success [18,22,26]. On the other hand, process strategies may affect many of these individual processes simultaneously [16]. Thus, understanding the biological limitations and application of an appropriate process strategy for addressing these limitations could be a better method for enhancement of the secretory expression of recombinant proteins.

This work has focused on investigating the biological constraints for the secretory production of recombinant streptokinase in *E. coli*. Streptokinase, a 47 kDa bacterial protein from *Streptococcus equisimilus*, is used as a thrombolytic agent in the treatment of acute myocardial infarctions following coronary thrombosis (for a recent review – see Ref [27]). Streptokinase has been cloned and expressed in *E. coli* [28] as a secretory as well as an intracellular product (both soluble and insoluble) [29–31]. Extensive proteolytic degradation of the secreted recombinant streptokinase has been reported [30,32]. Ramalingam et al. [32] have observed severe growth impairment and proteolysis of secretory recombinant streptokinase during the post-induction phase of fed-batch processes. Furthermore, the expression levels obtained were lower compared to some of the other recombinant proteins produced with the same signal peptide [4,32]. Attempts to mutate or change the signal peptide have also resulted only in partial enhancement of activity of the recombinant streptokinase produced by a secretory expression system [33].

In the present work, the problems in secretory expression of recombinant streptokinase were analyzed, along with a parallel analysis for the intracellular expression system. It was observed that accumulation of recombinant streptokinase in the cytoplasmic membrane (inner membrane) is a critical factor affecting the growth and productivity of the secretory expression system. This accumulation has been correlated to the up-regulation of an additional inner-membrane protease in the post-induction-phase. Partial characterization of the protease and its implications on the recombinant protein productivity are discussed. Reduction in cultivation temperature was employed as a simple process strategy to enhance the productivity of the secretory expression system. The enhancement was brought about by reduction in the accumulation level of the recombinant streptokinase in the inner membrane with a concomitant reduction in the activity of the inner-membrane protease.

2. Materials and methods

2.1. Bacterial strains and plasmids

E. coli BL21 (DE3) (Invitrogen) carrying T7 RNA Polymerase under the control of the *lac* promoter in the chromosome was used as a host for all the fermentation experiments. Plasmid pSSY4 containing the streptokinase gene, fused with an OmpA signal peptide under a T7 promoter, was used for periplasmic expression of recombinant streptokinase [30]. A pRSETB based expression vector was used for the intracellular expression of recombinant streptokinase. The intracellular expression vector was constructed by recloning the streptokinase gene without the OmpA signal peptide, from the pSYY4 vector on to the pRSETB vector.

2.2. Media and culture conditions

E. coli cells were routinely grown and maintained in LB medium (Tryptone, 10 g/l; yeast extract, 5 g/l; NaCl, 10 g/l) containing 100 μg Ampicillin per ml at 37 °C. The media used for batch fermentation consisted of K_2HPO_4, 5 g/l; KH_2PO_4, 3 g/l; NaCl, 0.5 g/l; NH_4Cl, 1 g/l; yeast extract, 5 g/l; glucose, 5 g/l; $MgSO_4$, 0.5 g/l; and trace metal solution, 1 ml/l (trace metal solution $FeSO_4$, 100 mg/l; $Al_2(SO_4)_3{\cdot}7H_2O$, 10 mg/l; $CuSO_4{\cdot}H_2O$, 2 mg/l; H_3BO_3, 1 mg/l; $MnCl_3{\cdot}4H_2O$, 20 mg/l; $NiCl_2{\cdot}6H_2O$, 1 mg/l; $Na_2MoO_4{\cdot}2H_2O$, 50 mg/l; $ZnSO_4{\cdot}7H_2O$, 5 mg/l) [34].

All shake flask experiments were carried out at 200 rpm and 37 °C unless otherwise mentioned. Batch bioreactor cultivations were carried out in a 3.7 l fermentor (KLF 2000, Bioengineering AG, Switzerland) equipped with pH, temperature, antifoam and dissolved oxygen controllers. Air was sparged at a constant rate (0.8–1 vvm). Dissolved oxygen tension was maintained at 30% of air saturation by controlling the agitation rate through a controller. The pH was controlled at 7. Temperature was maintained constant at 37 °C. Polypropylene glycol (PPG) was used as antifoam agent and added when required through the controller. The cultures were induced at the mid-exponential phase around 0.6 OD in the shake flask experiments and around 3 OD in the batch bioreactor experiments. IPTG at different concentrations (from 0.25 to 1 mM) was used as inducer.

2.3. Cell fractionation

Cell fractionations were carried out as explained below:

- *Extracellular fraction*: Cells were harvested by centrifugation at 6000 × *g* for 10 min. The supernatant was used as the extracellular fraction.
- *Periplasmic fraction*: Cell pellet, obtained from the above fractionation, was washed with buffer and then re-suspended in ice-cold 1 M Tris-Cl, 2 mM EDTA (pH 9.0), kept on ice for 20 min and then centrifuged at 10,000 × *g* for 15 min at 4 °C. The supernatant is the periplasmic fraction [35].
- *Cytoplasmic fraction*: The pellet, obtained from the periplasmic fractionation, was re-suspended in phosphate buffer