Gene 511 (2012) 455–460

Contents lists available at SciVerse ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Short Communication

Effect of periplasmic expression of recombinant mouse interleukin-4 on hydrogen peroxide concentration and catalase activity in *Escherichia coli*

Elnaz Mehdizadeh Aghdam [a,1], Lena Mahmoudi Azar [a,1], Abolfazl Barzegari [b,1], Farrokh Karimi [c], Majid Mesbahfar [a], Naser Samadi [d], Mohammad Saeid Hejazi [a,d,*]

[a] Department of Pharmaceutical Biotechnology, Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran
[b] Research Center for Pharmaceutical Nanotechnology, Tabriz University of Medical Sciences, Tabriz, Iran
[c] Department of Biotechnology, Faculty of Science, Maragheh University, Maragheh, Iran
[d] Faculty of Advanced Biomedical Sciences, Tabriz University of Medical Sciences, Tabriz, Iran

ARTICLE INFO

Article history:
Accepted 5 September 2012
Available online 18 September 2012

Keywords:
Hydrogen peroxide
Catalase activity
Periplasmic expression
Escherichia coli
Recombinant protein

ABSTRACT

Oxidative stress occurs as a result of imbalance between generation and detoxification of reactive oxygen species (ROS). This kind of stress was rarely discussed in connection with foreign protein production in *Escherichia coli*. Relation between cytoplasmic recombinant protein expression with H_2O_2 concentration and catalase activity variation was already reported. The periplasmic space of *E. coli* has different oxidative environment in relative to cytoplasm and there are some benefits in periplasmic expression of recombinant proteins. In this study, hydrogen peroxide concentration and catalase activity following periplasmic expression of mouse IL-4 were measured in *E. coli*. After construction of pET2mIL4 plasmid, the expression of recombinant mouse interleukin-4 (mIL-4) was confirmed. Then, the H_2O_2 concentration and catalase activity variation in the cells were studied in exponential and stationary phases at various ODs and were compared to those of wild type cells and empty vector transformed cells. It was revealed that empty vector introduction and periplasmic recombinant protein expression increased significantly the H_2O_2 concentration of the cells. However, the H_2O_2 concentration in mIL-4 expressing cells was significantly higher than its concentration in empty vector transformed cells, demonstrating more effects of recombinant mIL-4 expression on H_2O_2 elevation. Likewise, although catalase activity was reduced in foreign DNA introduced cells, it was more lowered following expression of recombinant proteins. Correlation between H_2O_2 concentration elevation and catalase activity reduction with cell growth depletion is also demonstrated. It was also found that recombinant protein expression results in cell size increase.

1. Introduction

Exceeding level of reactive oxygen species (ROS), such as superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2), and the other highly reactive oxygen radicals, is one of the most important stresses damaging all cell types (Cabiscol et al., 2000; Paradies et al., 2002). ROS are produced as a common by-product of aerobic life and consequent accumulation of ROS results in oxidation of various cell constituents as DNA (Wiseman and Halliwell, 1996), lipids and proteins (Stadtman, 1990). These caused oxidations considered as fundamental changes responsible for cell death (Boonstra and Post, 2004; Giannattasio et al., 2005). It is established that H_2O_2-induced oxidative stress results in the specific oxidation of thiol groups of proteins involved in detoxification of H_2O_2 and biosynthesis pathways such as thiolperoxidase, GTP-cyclohydrolase I, and the cobalamin-independent methionine synthase (MetE) (Leichert and Jakob, 2004). Inactivation of MetE, a highly expressed protein in *Escherichia coli*, by H_2O_2 is associated with a cellular methionine limitation imposed by oxidative stress (Hondorp and Matthews, 2004).

In *E. coli*, 87% of the total H_2O_2 is produced through respiratory chain. Most of the H_2O_2 in exponentially growing *E. coli* cultures is generated from superoxide ion ($2O_2^- + 2e^- + 4H^+ \rightarrow H_2O_2 + O_2$). Investigation on *E. coli* has shown that the generation of superoxide anion and hydrogen peroxide depends on the stage of culture development (Gonzalez-Flecha and Demple, 1995). In *E. coli*, H_2O_2 is removed by two kinds of catalases producing H_2O and O_2. These enzymes include hydroperoxidase I (HPI), existing during

Abbreviations: ROS, reactive oxygen species; mIL-4, mouse interleukin-4; MetE, cobalamin-independent methionine synthase; HPI, hydroperoxidase I; HPII, hydroperoxidase II; SOD, superoxide dismutase; OD, optical density; PCR, polymerase chain reaction; IPTG, isopropyl-beta-D-thiogalactoside; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid.

* Corresponding author at: Department of Pharmaceutical Biotechnology, Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran. Tel.: +98 411 3372256; fax: +98 411 3344798.
E-mail addresses: saeidhejazi@tbzmed.ac.ir, msaeidhejazi@yahoo.com (M.S. Hejazi).

[1] Authors contributed equally to this work and considered as co-first authors.

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http://dx.doi.org/10.1016/j.gene.2012.09.014

aerobic growth and transcriptionally controlled at various levels (Gonzalez-Flecha and Demple, 1997), and hydroperoxidase II (HPII) (Loewen et al., 1985; von Ossowski et al., 1991), which is induced during stationary phase.

All recombinant proteins are first synthesized in the cytoplasm and some of them are sent into the extracytoplasmic spaces for various applications (Baneyx, 1999; Cornelis, 2000). A common strategy to increase cell productivity and product quality is exporting products to the periplasm. Supplying of recombinant proteins into the periplasm has several benefits due to its oxidative environment leading to appropriate disulfide bond formation and consequent correct folding (Messens and Collet, 2006), less degradation due to presence of fewer proteases (Gottesman, 1996) and the easy extraction of final proteins (Anderluh et al., 2003). Furthermore, the translocation into the periplasm can result in easy purification by selective removal of outer membrane (Shokri and Larsson, 2004). The presence of superoxide dismutases containing copper plus zinc ions (Cu, Zn-SOD) (Benov and Fridovich, 1994) and KatG (HPI) enzymes metabolizing superoxide anion and hydrogen peroxide in the periplasmic space of *E. coli*, respectively, protects the environment from oxidative damage (Lushchak, 2011).

Introduction of foreign DNA and expression of recombinant protein in host organisms often change and impair the organism's normal metabolism and cell growth. This kind of metabolism alteration arises from the metabolic load (Glick, 1995). The metabolic load is considered as the main reason for cell growth depletion in recombinant cells. Production of adequate amounts of recombinant proteins is essential for small to large scale recombinant protein production. There are several approaches to improve recombinant protein production, such as selecting high level expression systems, optimizing expression conditions for improving protein solubility (Doonan, 1996) and optimization of media formulation (Broedel et al., 2001; Rathore et al., 2003). Additionally, understanding of the factors decreasing recombinant protein yield, plays a key role in the improvement of recombinant protein production. Therefore, determination of inhibiting factors during foreign protein production and resolving their inhibitory effects could improve the yield of the production theoretically. In this path, we studied the effect of cytoplasmic expression of recombinant proteins on H_2O_2 concentration and catalase activity. Our results showed a significant elevation in hydrogen peroxide concentration as the most stable component of ROS and reduction of catalase activity as an important H_2O_2 decomposer (Hejazi et al., 2009). This could be considered as a limiting factor in production of recombinant protein. Considering the advantages of periplasmic expression of foreign proteins and consequently the high interest in production of recombinant proteins in periplasmic space, we aimed to investigate the effect of periplasmic expression of recombinant mouse IL-4 as a non-enzymatic, nontoxic and inert protein on H_2O_2 concentration and catalase activity in the recombinant cells. Moreover, correlation between H_2O_2 concentration and catalase activity following recombinant protein expression with cell growth was studied. The special emphasis is given to the comparison of the alteration in H_2O_2 concentration and catalase activity among recombinant protein expressing, foreign DNA introduced and wild type cells.

2. Materials and methods

2.1. Molecular biology reagents

Primers mIL4F (5′-TAC GGA TCC CCA TAT CCA CGG ATG C-3′) and mIL4R (5′-GGC CTC GAG CTA CGA GTA ATC CAT-3′), supplied by MWG-Eurofine Operon, were used as forward and reverse primers for amplification of mIL-4 encoding DNA. Luria-Bertani (LB) (Bactotryptone 10 g/l, yeast extract 5 g/l, and NaCl 10 g/l with pH 7.0, all purchased from Merck-Germany) was used as culture medium, and ampicillin (100 μg/ml) was added into the medium when it was required to maintain selection pressure. Retroviral based pWZLIL/4B7M expression plasmid (a kind gift from Dr. Joop Gäken King's College London, London) was used as the DNA template for PCR amplification of mature mIL-4 (composed of 120 amino acids) encoding DNA. pTZ57R/T was used as cloning vector and pET-22b(+) was used as prokaryotic periplasmic expression vector under the control of inducible T7 promoter. pET-22b(+) empty vector was also used as the negative control.

2.2. PCR amplification of mIL-4 DNA and construction of pET2mIL4 vector

PCR amplification of mIL-4 DNA was carried out using mIL4F and mIL4R primers. These primers created *Bam*HI and *Xho*I restriction sites at 5′ and 3′ ends of the amplified DNA, respectively. PCR amplification was performed under the following conditions: 94 °C for 5 min, followed by 35 cycles of 94 °C for 45 s, 54 °C for 30 s and 72 °C for 30 s and final extension was performed at 72 °C for 10 min. Mouse mature IL-4 encoding DNA was amplified using PCR technique and the amplification result was evaluated using gel electrophoresis. The presence of a DNA band with about 360 bp size confirmed the amplification of mIL-4 encoding DNA. The mIL-4 encoding DNA was cloned into pTZ57R/T vector and then subcloned into pET-22b(+) using *Xho*I and *Bam*HI restriction sites. The success of cloning was evaluated by double digestion of the resultant plasmids with *Xho*I and *Bam*HI enzymes. The release of a DNA band with about 360 nucleotides represented the presence of mIL-4 encoding DNA inside the vector. The constructed plasmid was called pET2mIL4, in which IL-4 DNA is driven by inducible T7 promoter. All DNA manipulation procedures were conducted based on common molecular biology protocols. All bacterial cultures in the present study were conducted in aerobic condition.

2.3. Evaluation of mIL-4 expression

E. coli BL21(*DE3*) competent cells were transformed using heat shock technique. Bacterial transformants were cultured on LB supplemented with ampicillin and isopropyl-beta-D-thiogalactoside (IPTG) was added into the medium as the expression inducer with final concentration of 0.5 mM at desired OD. The bacterial cells were harvested 2 h after IPTG treatment and total and periplasmic proteins were extracted with modified boiling method (Doonan, 1996) and osmotic shock procedure (Rathore et al., 2003), respectively. The extracts were analyzed by SDS-PAGE methods. SDS-PAGE was performed by a modified method (12 separating gel, 5% stacking gel) and gels were stained with comassie brilliant blue. In this experiment, wild type (non-transformed) and non-induced transformed cells were used as negative controls.

2.4. Growth curve

For drawing growth curve, the bacterial cells were cultured overnight in LB. OD of the overnight grown cultures was adjusted at 1, diluted 1/100 (v/v) and then the cells were incubated at the same temperature. The media were supplemented with IPTG with final concentration of 0.5 mM at OD 0.5. The cultures' OD was measured spectrophotometrically at 600 nm every half hour for 12 h.

2.5. Cell extraction for H_2O_2 assay

OD of the overnight culture of the cells was adjusted at 1, diluted 1/100 (v/v) and incubated for further growth. Then, 1.5 ml of bacterial culture at ODs 0.65, 0.7, 0.8 and 1.2 was centrifuged at 13,000 rpm for 15 min at room temperature. The pellet was resuspended in 5 ml of 0.1% (w/v) trichloroacetic acid in an ice bath, sonicated at 22 kHz