Protein Expression and Purification 118 (2016) 105–112

Contents lists available at ScienceDirect

Protein Expression and Purification

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

High-efficiency secretory expression of human neutrophil gelatinase-associated lipocalin from mammalian cell lines with human serum albumin signal peptide

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ARTICLE INFO

Article history:
Received 2 July 2015
Received in revised form
19 October 2015
Accepted 21 October 2015
Available online 27 October 2015

Keywords:
Neutrophil gelatinase-associated lipocalin (NGAL)
Signal peptide
Secretory expression
Mammalian cells
Acute kidney injury (AKI)

ABSTRACT

Human neutrophil gelatinase associated lipocalin (NGAL) is a secretory glycoprotein initially isolated from neutrophils. It is thought to be involved in the incidence and development of immunological diseases and cancers. Urinary and serum levels of NGAL have been investigated as a new biomarker of acute kidney injury (AKI), for an earlier and more accurate detection method than with creatinine level. However, expressing high-quality recombinant NGAL is difficult both in *Escherichia coli* and mammalian cells for the low yield. Here, we cloned and fused NGAL to the C-terminus of signal peptides of human NGAL, human interleukin-2 (IL2), gaussia luciferase (Gluc), human serum albumin preproprotein (HSA) or an hidden Markov model-generated signal sequence (HMM38) respectively for transient expression in Expi293F suspension cells to screen for their ability to improve the secretory expression of recombinant NGAL. The best results were obtained with signal peptide derived from HSA. The secretory recombinant protein could react specifically with NGAL antibody. For scaled production, we used HSA signal peptide to establish stable Chinese hamster ovary cell lines. Then we developed a convenient colony-selection system to select high-expression, stable cell lines. Moreover, we purified the NGAL with Ni-Sepharose column. The recombinant human NGAL displayed full biological activity. We provide a method to enhance the secretory expression of recombinant human NGAL by using the HSA signal peptide and produce the glycoprotein in mammalian cells.

1. Introduction

Acute kidney injury (AKI) refers to the rapid decrease of kidney function caused by numerous primary or secondary factors; it leads to kidney failure [1]. AKI is an important public health issue because of the substantial morbidity and mortality in critically ill patients [2,3]. Early diagnosis and treatment are important for the reversal of AKI. However, the current diagnosis of AKI depends on elevated blood urea nitrogen and creatinine levels, which lack immediacy, sensitivity and specificity [4].

Human neutrophil gelatinase-associated lipocalin (NGAL) was found in 1993, covalently bound to gelatinase in human neutrophils [5]. The glycoprotein shows low-level expression in various tissues such as kidney, prostate, and liver and in epithelial cells [6]. NGAL level is upregulated in cells under various pathologic states such as ischemia [7]. Increased urinary and serum levels of NGAL were observed in patients with renal failure [6,8]. NGAL is specifically induced in AKI and rapidly secreted into blood and urine within 2 h [9]. A clinical study monitoring 635 patients found urine NGAL with high sensitivity (90%) and specificity (99%) in the diagnosis of AKI [10]. Urinary NGAL level is more accurate than serum creatinine

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http://dx.doi.org/10.1016/j.pep.2015.10.012
1046-5928/

Abbreviations

AKI	Acute kidney injury
NGAL	neutrophil gelatinase-associated lipocalin
GS	glutamine synthetase
MSX	L-Methionine sulfoximine
PBS	Phosphate buffer saline
PBST	PBS with 0.02% Tween-20
PVDF	polyvinylidene difluoride
CHO	Chinese hamster ovary
IL2	interleukin-2
Gluc	gaussia luciferase
HSA	human serum albumin preproprotein
HMM38	hidden Markov model-generated signal sequence

level in AKI [11]. NGAL has attracted much attention as a new biomarker for the early diagnosis of AKI [12]. The establishment of an NGAL immunological detection method would be helpful for AKI diagnosis, treatment and prognosis prediction.

Therefore, the efficient preparation of NGAL protein is necessary for development of AKI early diagnosis methods and other biological application study. Usually, recombinant production systems offer higher productivity than non-recombinant production systems [13]. Several methods have been developed to produce recombinant human NGAL in *Escherichia coli* [14], Baculovirus [15,16], and human embryonic kidney 293T (HEK293T) cell [17] expression systems. Expressing recombinant NGAL in prokaryotic systems has clear disadvantages such as insolubility, lack of protein modification and insufficient purity, and the purification process is time-consuming. Problems also exist in eukaryotic systems because of low protein yield and high costs.

To overcome these problems with NGAL, we constructed expression clones to improve the secretory expression of NGAL in mammalian cells such as Expi293F and Chinese hamster ovary (CHO) cells. Signal peptides including native signal peptide of NGAL and signal peptides of human interleukin-2 (IL2), gaussia luciferase (Gluc), human serum albumin preproprotein (HSA) and an hidden Markov model-generated signal sequence (HMM38) were evaluated for their ability to enhance the secretory expression of NGAL in mammalian cells.

2. Materials and methods

2.1. Cell culture

The Expi293F cell line was purchased from GIBCO (Catalog no. A14527). The CHO cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Both cell lines were adapted to serum-free culture media and growth in suspension. Cells were cultured in roller bottles under standard procedures at 120 rpm and 37 °C with 8% CO_2.

2.2. Construction of NGAL fusion expression vectors

The sense and antisense oligonucleotides of five signal peptides in Table 1 (Genscript Biotechnology, Piscataway, NJ, USA) were annealed to produce fragments containing a HindIII sticky end at the 5′ end, a kozak sequence, a signal sequence, a 6 × His sequence, a 2 × stop codon and an XbaI sticky end at the 3′ end. The annealed products were then ligated into the HindIII-XbaI sites of the glutamine synthetase (GS)-based pcDNA vector (GS-pcDNA), which was constructed by replacing the neo gene in pcDNA3.1(+) mammalian-expressing vector with glutamine synthetase gene. The resulting plasmids were named sp-pcDNA, with five different signal sequences (spNative-pcDNA, spIL2-pcDNA, spGLuc-pcDNA, HMM38-pcDNA and spHSA-pcDNA).

The complete coding sequence of human NGAL (NP_005555.2) was synthesized by Genscript Biotechnology and cloned into the pUC57 plasmid. The open reading frame of NGAL was PCR-amplified and cloned into the PCR-linearized sp-pcDNA between the signal fragment and 6 × His tag by use of the ClonExpress II one-step cloning kit (C212-01, Vazyme, Piscataway, NJ, USA) to obtain the sp-NGAL-pcDNA plasmids (spNative-NGAL-pcDNA, spIL2-NGAL-pcDNA, spGLuc-NGAL-pcDNA, HMM38-NGAL-pcDNA, spHSA-NGAL-pcDNA) respectively (Fig. 1). PCR primers are listed in Table 2.

2.3. Transient expression in Expi293F cells

The sp-NGAL-pcDNA plasmids were transfected into 7.5×10^7 Expi293F cells in 30 ml Expi293 Expression Medium (A14351-01, GIBCO BRL) by use of the ExpiFectamine 293 Transfection Kit (A14524, GIBCO BRL) and Opti-MEM Reduced Serum Medium (31985-062, GIBCO BRL) for 16–18 h, then Enhancer 1 and Enhancer 2 was added. The cultures were expanded for 7 days to allow for protein expression. During cultivation, cell density was monitored daily by using blood cell counters, when the cell density starting falling, 3 mM L-Glutamine (21051, GIBCO) and 3 g/L Cell Boost 5 (SH30865, Hyclone) were added into the culture. Cell viability was determined by Trypan Blue exclusion assay. The culture supernatant was harvested on day 7.

2.4. CHO cell transfection and screening for stable cell lines

The screening of stable cell lines is based on the balance between the expression of the glutamine synthetase introduced by the expression plasmid and the addition of methionine sulfoximine (MSX), an inhibitor of glutamine synthetase.

To obtain high-level expression stable cell line, the plasmid spHSA-NGAL-pcDNA was electrically transfected into 1×10^7 CHO cells by use of Gene PulserXcell (BIO-RAD); cells were then transferred to 10 ml CDM4 medium (SH30558.02, HyClone) without L-glutamine and MSX. After recovery of the cell viability, MSX was added into the culture medium gradually increasing to a final concentration of 50 μg/ml according to the cell density and viability.

Approximately 15 days after MSX selection, about 10,000 viable cells were immobilized and incubated in CloneMatrix semi-solid medium MCWO413B (K8510, Molecular Devices) containing 12 μg/ml FITC-labeled NGAL antibody, a mouse polyclonal antibody prepared with NGAL antigen and used for screening high-level expression cell colonies. Cells were incubated to form colonies under 37 °C with 5% CO_2. After about 15 days, secreted protein retained near its associated colony could be visualized by the immunoprecipitate formed by interaction between recombinant protein and FITC-labeled NGAL antibody. The immunoprecipitates were bright-green dots around the colony. High expression colonies with brighter dots surrounding were chosen for further culture and evaluation.

2.5. Fed-batch cultivation

The selected colonies were further evaluated for their ability of expressing the NGAL protein by fed-batch or batch cultivation. For the fed-batch cultivation, colonies were gradually expanded in shaken flasks containing 10 ml CDM4 medium, the Cell Boost 5 (3 g/