Biochemical Engineering Journal 62 (2012) 86–91

Contents lists available at SciVerse ScienceDirect

Biochemical Engineering Journal

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

Production, purification and cytotoxity of soluble human Fas ligand expressed by *Escherichia coli* and *Dictyostelium discoideum*

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ARTICLE INFO

Article history:
Received 31 January 2011
Received in revised form 1 January 2012
Accepted 3 January 2012
Available online 14 January 2012

Keywords:
Human Fas ligand
Escherichia coli
Dictyostelium discoideum
Soluble production
Affinity chromatography
Cytotoxic activity

ABSTRACT

Human Fas ligand (hFasL) is a type II membrane protein that induces apoptosis in the Fas-bearing cells. Its special biological activity has the potential for the therapeutic use as an anti-cancer agent directed at enhancing apoptosis in tumor cells. In this study *Escherichia coli* and eukaryotic *Dictyostelium discoideum* were used to produce a soluble form of hFasL in large amounts. An expression vector for hFasL production in *E. coli* was constructed based on plasmid pET32a(+). By cultivation of the hFasL-producing *E. coli* clone on LB medium and induction with IPTG, a hFasL concentration of 1.0 mg L^{-1} was achieved. *D. discoideum* strain AX3-hFasL-H was cultured in a conventional stirred bioreactor on an improved synthetic medium using a simple fed-batch strategy, and cell densities of up to 8.3×10^7 cells/mL and a maximum hFasL concentration of 420 μg/L were obtained. Using Ni-NTA affinity chromatography purification, two kinds of recombinant hFasLs from *E. coli* and *D. discoideum* were purified with a purity of 94% and 90%, respectively. They showed similar biological activities in inducing apoptosis in Fas-expressing cells.

1. Introduction

Human Fas ligand (hFasL) is a 37 kDa transmembrane glycoprotein belonging to the tumor necrosis factor (TNF) family. Loss of hFasL leads to lymphoproliferative and autoimmune diseases [1,2]. Human FasL induces apoptosis by binding to human Fas receptors on the target cells for eliminating the emerging harmful cells, such as cancerous cells and virus/bacteria infected cells [3–5]. The primary structure of hFasL molecule (281 aa) can be divided into three structural domains, i.e., the intracellular domain (80 aa), transmembrane domain (22 aa) and extracellular domain (179 aa) [6]. The extracellular domain of hFasL possesses three putative *N*-glycosylation sites at aspartic acid residues 184, 250 and 260, which may contribute to the efficient secretion of hFasL, but not to its biological activity [7].

Fas ligand also exists in a soluble form, which is biologically active and can compete with the membrane-bound form to regulate apoptosis [8]. A soluble form of recombinant hFasL has been prepared from mammalian cells [7,9,10], and insect cells [11], *Dictyostelium discoideum* amoeba [12], yeast [13,14] and bacteria [15], where in all cases the genes derived from the extracellular region genes of hFasL were employed. Among these hosts, *D. discoideum* has a post-translational mechanism which can produce glycoproteins with a glycosylation pattern that is more comparable to the normal mammalian one. The extracellular recombinant hFasL expressed by *D. discoideum* has been proved to be biologically active with or without the 6-his tag [12,16]. A concentration of up to 157 μg/L 6-his tagged hFasL was achieved on the standard complex medium HL-5C [16].

Due to misfolding and the formation of inclusion bodies in overexpression of oligomers or the low quantity of expressing complex glycoproteins, it is difficult to obtain the recombinant hFasL in *Escherichia coli* in soluble and active form [15]. Since the effect of gene fusion on expression efficiency was reported [15], in this study a recombinant vector based on pET32a(+) with Trx-tag for expression of a fusion form of soluble hFasL was constructed to overcome these problems. Production and biological activities between recombinant hFasLs expressed by *E. coli* and *D. discoideum* were compared, respectively. The results indicate that a relatively large amount of biologically active hFasL may be prepared from *E. coli* and *D. discoideum* in soluble form.

2. Materials and methods

2.1. Microbial strains

Dictyostelium discoideum strains AX3-pLu8 (*D. Discoideum* AX3 with hFasL expression plasmid pMB74-hFasL) [17] and

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doi:10.1016/j.bej.2012.01.002

AX3-hFasL-H (in short AX3-FH) (*D. Discoideum* AX3-pLu8 with C-terminal 6-his tag) [16] were used in this study.

E. coli strain DH 5α (Invitrogen, Paisley, UK) was used as a bacterial host for plasmid construction. Protein production was performed in the *E. coli* strain BL21(DE3) (Novagen, Madison, WI, USA).

2.2. Media

Yeast extract was purchased from Bioferm (Waldmünchen, Germany). Bactotryptone was supplied by Difco (Detroit, MI, USA). Amino acids were from Ajinomoto (Tokyo, Japan). All other chemicals were obtained from Sigma (St. Louis, Missouri, USA) or Merck (Darmstadt, Germany) and were of analytical grade.

Recombinant *E. coli* cells were cultivated on Luria–Bertani (LB) medium consisting of 5 g/L yeast extract, 10 g/L tryptone and 10 g/L NaCl.

A newly developed complete synthetic medium (improved SIH medium) based on the SIH synthetic medium formulated by Han et al. [18] with slightly changed amino acid, salt and vitamin compositions was employed for the production of soluble hFasL by *D. discoideum*. The composition of the improved SIH medium is given in Table 1, for convenience.

The improved SIH medium was prepared on the basis of four solutions containing either amino acids, vitamins, salts, or trace elements. The amino acid solution was prepared with 4 folds, the vitamin solution with 20 folds, the salt solution with 50 folds, and the trace element solution with 10,000 folds of the concentration of the final medium. Therefore, 250 mL of the amino acid solution, 50 mL of the vitamin solution, 20 mL of the salt solution and 0.1 mL of the trace element solution were added together with glucose, the phosphate salts and antibiotics (50 mg dihydroxystreptomycin sulfate and 5 mg geneticin), and filled up with distilled water to 500 mL. The pH was adjusted to 6.5 by adding dilute HCl or NaOH solutions. Finally, the volume of the medium was brought to 1 L by adding distilled water. The medium was sterilized by filtering through a membrane filter system Sartobran 300 (Type: 5231307-H5-00B, Sartorius, Göttingen, Germany).

For *D. discoideum* spore activation and comparison, the complex axenic HL-5C medium [12] containing 10 g/L glucose, 5 g/L yeast extract, 2.5 g/L bactotryptone, 2.5 g/L casein peptone, 5 g/L proteose peptone, 1.2 g/L KH_2PO_4, and 0.35 g/L Na_2HPO_4 at pH 6.5 was also used.

2.3. Construction of E. coli expression vector

The expression vector was constructed using standard laboratory techniques [19]. A 420 bp DNA fragment encoding the extracellular region of hFasL (amino acids 141–281) was PCR-amplified from plasmid pMB74-hFasL with the forward primer 5′-CTCGA*GGATCC* TGGGCTTCTAAGGAGCTGAGGAAAGTGG-3′ and the reverse primer 5′-CTGCA*AAGCTT* TTAGAGCTTATATAAGCCGAA-3′, respectively (*Bam*H I and *Hin*d III restriction sites are italicized). The PCR product was cleaved with *Bam*H I and *Hin*d III and inserted into the corresponding sites of pET32a(+) (Novagen, Madison, WI, USA), resulting in the expression vector pET32a-hFasL. The recombinant plasmid was transformed into *E. coli* DH5α and checked through DNA sequencing. Finally, pET32a-hFasL was extracted from the cell culture and transformed into *E. coli* BL21(DE3) for the protein expression. The generated strain was screened by ampicillin selection, and called BL21(DE3)-pET32a-hFasL.

For negative control, *gld*, a mutant and inactive form of hFasL, was also generated by PCR site-directed mutagenesis using the forward primer described above and a mutated reverse primer 5′-CTGCA*AAGCTT*TTAGAGCTTATATAAGCCCAAAAACGT-3′ (*Hin*d III restriction sites is italicized and mutated site underlined). The product was digested with *Bam*H I and *Hin*d III and inserted into pET32a(+) to generate pET23a-*gld*, and the recombinant plasmid was checked through DNA sequencing. The recombinant plasmid pET23a-*gld* was then transformed into *E. coli* BL21(DE3), yielding recombinant strain BL21(DE3)-pET32a-*gld*.

2.4. Shake flask cultivation

The *E. coli* stain BL21(DE3)-pET32a-hFasL was grown in LB medium with ampicillin (50 μg/mL) at 37 °C overnight. The cells were then inoculated into 200 mL of fresh LB medium in 1000 mL baffled shake flasks and allowed to grow to an optical density ($OD_{600\,nm}$) of 1.0 on a rotary shaker at 37 °C and 150/min. Protein production was induced by adding IPTG (isopropyl-β-thiogalactopyranside) to a final concentration of 0.2 mM. After cultivation at 30 °C for 3 h, the cells were harvested by centrifugation at 1000 × *g* and the pellets were stored at −70 °C.

The *D. discoideum* expression strain, AX3-FH, was inoculated into 30 mL HL-5C or improved SIH medium in 300 mL Erlenmeyer flasks, or into 100 mL improved SIH medium in 500 mL Erlenmeyer flasks at an initial cell concentration of 0.5–1 × 10^5 cells/mL and incubated for 4–5 days at 21–22 °C on a rotary shaker at a rotational frequency of 150/min. For all experiments 10 μg/mL G418 was added.

Table 1
Composition of the improved synthetic SIH medium for *Dictyostelium discoideum*.

Substances	Conc.	Substances	Conc.	Substances	Conc.
– **Amino acids**	mmol L^{-1}	– **Vitamines**	mg L^{-1}	– **Salts**	mmol L^{-1}
L-Arginine	3.8	Biotin	0.010	$NaHCO_3$	0.2
L-Asparagine	2.3	Cyanocobalamin	0.005	NH_4Cl	1.0
L-Aspartic acid	1.1	Folic acid	0.20	$CaCl_2$	0.02
L-Cysteine·HCl	2.5	Lipoic acid	0.40	$FeCl_3$	0.10
Glycine	12.0	Riboflavin	2.5	$MgCl_2$	1.29
L-Glutamic acid	3.7	Thiamine·HCl	0.60		
L-Histidine	1.8				
L-Isoleucine	4.6				
L-Leucine	6.9	– **Phosphate salts**	mmol L^{-1}	– **Trace elements**	μmol L^{-1}
L-Lysine·HCl	10.0	KH_2PO_4	8.82	Na_2EDTA	13
L-Methionine	0.8	NaH_2PO_4	2.47	H_3BO_3	1.8
L-Phenylalanine	3.3			$CoCl_2$	0.7
L-Proline	7.0			$CuSO_4$	0.6
L-Threonine	4.2	– **C-source**	mmol L^{-1}	$(NH_4)_6Mo_7O_{24}$	0.08
L-Tryptophan	2.1	Glucose	66.0	$MnCl_2$	2.6
L-Valine	6.0			$ZnSO_4$	8