Overproduction, purification and characterization of human interferon alpha2a-human serum albumin fusion protein produced in methilotropic yeast *Pichia pastoris*

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Abstract. Human interferon alpha2a (hIFNα2a) is a therapeutic protein that used in cancer and hepatitis B/C therapy. The main problem of using hIFNα2a is its short elimination half life due to its low molecular weight. Development of higher molecular weight protein by albumin fusion technology is a rational strategy to solve the problem. In our previous research we constructed an open reading frame (ORF) encoding hIFNα2a-human serum albumin (HSA) fusion protein that expressed in *Pichia pastoris* (P. pastoris) protease deficient strain SMD1168. This research was performed to overproduce, purify and characterize the fusion protein. To overproduce the protein, cultivation was performed in buffered complex medium containing glyserol (BMGY) for 24 h and protein overproduction was applied in buffered complex medium containing methanol (BMMY) for 48 hours at 30°C. The fusion protein was purified by blue sepharose affinity chromatography. Molecular weight characterization by SDS PAGE corresponds with its theoretical size, 85 kDa. Western blot analysis demonstrated that the fusion protein was recognized by anti hIFNα2a and anti HSA monoclonal antibody as well. Amino acid sequence of the fusion protein was determined by LC MS/MS2 mass spectrometry with trypsin as proteolitic enzyme. There were three fragments that identified as hIFNα2a and seven fragments that identified as HSA. Total identified amino acids were 150 residues with 20% coverage from total residues. To conclude, hIFNα2a-HSA fusion protein was overproduced, purified and characterized. Characterization based on molecular weight, antibody recognition and amino acid sequence confirmed that the fusion protein has correct identity as theoretically thought.

1. Introduction

hIFNα2a is a cytokine that consists of 165 amino acids with 19 kDa in size. It has wide biological activity such as antivirus, antiproliferation as well as immunomodulation [1]. Recombinant hIFNα2a has already used as therapeutic protein in hepatitis B/C and several cancer treatments since 1986 [2]. The major problem of using hIFNα2a for patient treatment is short elimination half life due to rapid renal clearance. As low molecular weight protein, major elimination route of the protein in the body is through renal filtration system by glomerulus. The consequence of short half-life time is high

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frequency treatment to maintain the effective concentration of the protein. The treatment frequency for hepatitis B or C is three times per week for 24 to 48 weeks and for oncological indication is daily from several months to a year or longer [3].

The strategies to improve the elimination half-life of rhIFNα2a have been performed with several approaches. The most successful is higher molecular weight modification by pegylation. The commercial pegylated protein PEGASYS (Hoffman La Roche, UK) with 40 kDa PEG molecule has 70 hour of elimination half life that decreases the therapy frequency become only once a week [4]. Albumin-fusion technology is a rational, simple and flexible alternative strategy to increase the protein molecular weight through a fusion between rhIFNα2a and human serum albumin (HSA). HSA has a 9 day long half-life, widely distributed in the body and not immunogenic. Moreover it may improve the solubility and stability of the protein [5].

The albumin fusion technology widely used as fusion partner to extend in vivo half life of protein therapeutics at its N terminal or C terminal. Zhao et al. reported that HSA fusion at the N-terminal of rhIFNα2b caused instability due to the disruption of disulfide bond that formed by cysteine at positions 1 to 98. The heterogeneity can be avoided by adding a linker between the two proteins or altering the orientation of HSA fusion at the N-terminal into C-terminal [6]. The study of rhIFNα2b-HSA fusion protein reported the improvement of elimination half-life from 5 hours into 159 hours that decrease therapy frequency become once in two week [5]. In our previous research we synthesized an ORF encoding rhIFNα2a-HSA fusion protein. The constructed ORF in frame with α-factor signal sequence to obtain extracellular fusion protein. This research was purposed to overproduce, purify and characterize the rhIFNα2a-HSA fusion protein.

2. Materials and Methods

2.1 Overproduction

Overproduction was performed using shake flask method with 50 mL of BMMY media. Single colony was grown overnight in 25 ml BMGY medium at 30°C and 250 rpm until log phase (OD600 = 2-6). The culture was centrifuged at 1500 x g for 5 min. The pellet was resuspended in 50 ml BMMY medium (OD600 = 1.0 containing 1.5% methanol as inducer). Protease inhibitor cocktail (Roche) was added to overcome protein degradation. The 48h harvesting time was carried out by repeating the induction after 24 h cultivation time. Harvesting was performed by centrifugation at 1500 x g for 5 minutes at room temperature to collect supernatant.

2.2 Purification

Supernatant containing fusion protein was concentrated 10X (v/v) using tangential filtration system with 10 kDa molecular weight membrane cut off. Concentrated supernatant was purified using affinity chromatography method by blue sepharose. The concentrated fraction was applied into prewashed blue sepharose resin (GE Healthcare). The prewashed was applied using PBS 1x pH 7.0 as wash buffer. After 3 times sample reloaded, the resin was washed 5 times using wash buffer, then eluted with 5 mL elution buffer containing PBS 1x pH 7.0 and 1.5 M NaCl. The purified protein was filtered by Amicon® (Millipore) with 10kDa molecular weight cut off.

2.3 Characterization

Protein molecular weight was characterized by SDS-PAGE method with 10% (w/v) of polyacrylamide gel and coomassie blue staining solution (BioRad). Western blot used to identify the protein based on specific monoclonal antibody recognition. The primary antibodies was mouse anti IFNα2 (Merck 407290-500 UGCN, Germany) with 1: 1000 dilution or mouse anti HSA (Sigma, USA) with 1:1000 dilution. The secondary antibody was 1: 7500 dilution of anti mouse IgG alkaline phosphatase conjugate (Promega, USA) with NBT/BCIP detection (Merck, Germany). Amino acid sequence was determined by LC MS/MS2 method (Proteomic Inc). The samples prepared from coomassie blue stained gel slice.
3. Result and Discussion

The overproduction of fusion protein in the *P. pastoris* consisted of biomass increment in BMGY media and protein production in BMMY media. Glycerol in the BMGY media is the main carbon source and repressor of alcohol oxidase 1 (*AOX1*) promoter. BMMY media provides methanol as carbon source [7]. The concentration of methanol in the medium determined the efficiency of the recombinant protein production. Overproduction in *P. pastoris* depends on biosynthesis of alcohol oxidase (AOX) which is regulated by *AOX1* promoter. We constructed an ORF encoding fusion protein in the downstream of *AOX1* promoter, therefore its expressions would be in line with AOX. *P. pastoris* can produce proteins intracellularly and extracellularly. In this study the ORF constructed fusion protein contains α-factor preprotein signal peptides, so the fusion protein produced extracellularly.

One of disadvantages high cell density overproduction is protein instability due to protease activities. Combination of high density culture and small percentage cell lysis in the media may cause some proteins rapidly degraded. The protease such as proteinase A and proteinase B, carboxylypeptidase and aminopeptidase are major known proteases that induce protein degradation in *P. Pastoris* [8]. Several strategies to inhibit proteolysis activity have been reported, such as protein engineering [9], optimization of fermentation parameters (pH, temperature and growth rate), modification of culture media composition (rich media, additional amino acid or peptone, lowering salt concentration as well as soytone addition) and the use of protease deficient strain [10]. In this study we used protease deficient strain SMD1168 that lack of proteinase A activity. Protease inhibitor also used in overproduction media to reduce proteolysis as well. After 48h of overproduction, the supernatant containing fusion protein was collected, purified and characterized.

Fusion protein purification performed by affinity chromatography using blue sepharose (GE healthcare) resin that consists cross-linked 6% agarose beads modified with Cibacron Blue 3G covalently attached by the triazine coupling method. The blue dye binds many proteins including albumin and interferon. Molecular weight characterization of purified fusion protein informed that the protein was 85 kDa in size which corresponds with its theoretical size (Figure 1). Further characterization using anti HSA and anti hIFNα2 monoclonal antibodies showed that the protein was recognized by both antibodies (Figure 2). This result strongly indicated the correct identity of fusion protein. Amino acid sequence confirmed the antibody based characterization with ten identified peptides. By using trypsin as proteolitic enzyme, there were three fragments that identified as hIFNα2a and seven fragments that identified as HSA. Total identified amino acid were 150 residues with 20% coverage from total residues (Figure 3).

![Figure 1. Molecular weight analysis of fusion protein by SDS PAGE, 1=eluate 1; 2=eluate 2; 3=eluate 3.](image-url)
Figure 2. Monoclonal antibody recognition-based analysis of fusion protein, A= using anti hIFNα2 antibody, B= using anti HSA antibody

Figure 3. Amino acid sequence determination of fusion protein. hIFNα2a amino acids shown in blue and HSA amino acids shown in red.

4. Conclusions
Fusion protein has been overproduced, purified as well as characterized. The identified fusion protein has 85 kDa molecular weight in size and confirmed as fusion protein between hIFNα2a and HSA.

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