PUBLICATIONS
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Process optimization of constitutive human granulocyte–macrophage colony-stimulating factor (hGM-CSF) expression in Pichia pastoris fed-batch culture

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Abstract Human granulocyte–macrophage colony-stimulating factor (hGM-CSF) is a therapeutically important cytokine that is poorly expressed because of its toxic effects on the host cells. Extracellular expression of hGM-CSF was obtained by cloning its gene in Pichia pastoris under the constitutive glyceraldehyde-3-phosphate dehydrogenase (GAP) promoter with an N-terminal α peptide sequence for its extracellular production. The clones obtained were screened for a hyper producer following which media and cultivation conditions were optimized in shake flasks. Batch and fed-batch studies were performed in a bioreactor where different feed compositions were fed exponentially to obtain high biomass concentrations. Feeding of complex media allowed us to maintain a high specific growth rate of 0.2 h⁻¹ for the longest time period, and a final biomass of 98 g DCW/l was obtained in 34 h. Product formation was found to be growth associated, and the product yield with respect to biomass (YP/X) was ~2.5 mg/g DCW. The above fed-batch strategy allowed us to obtain fairly pure glycosylated hGM-CSF at a final product concentration of 250 mg/l in the culture supernatant with a high volumetric productivity of 7.35 mg l⁻¹ h⁻¹.

Introduction

Human granulocyte–macrophage colony-stimulating factor (hGM-CSF) is a hematopoietic growth factor that mediates the differentiation and proliferation of granulocyte and macrophage colonies in the bone marrow (Metcalf 1985). There is a lot of clinical interest in this lymphokine due to its ability to stimulate granulocyte and macrophage colony formation in patients who are immuno-suppressed because of diseases or from receiving chemotherapy or radiation therapy. This lymphokine is used in the treatment of myeloid leukemia, neutropenia and aplastic anemia and greatly reduces the risk of infection associated with bone marrow transplantation by accelerating neutrophil formation (Hussein et al. 1995; Armitage 1998). The toxicity of hGM-CSF towards its host is the major bottleneck hampering overexpression. In E. coli, cytoplasmic expression of recombinant hGM-CSF leads to stoppage of growth upon induction, and cell lysis is typically observed, the yields therefore are extremely poor. However, periplasmic expression using SP1 and SP3 signal peptide increased the expression level to 0.45 mg ml⁻¹ OD⁻¹ (Berges et al. 1996). Supplementation of a tRNA for a rare codon, which codes for Arginine, led to a slight improvement in production levels (Hua et al. 1994), whereas fusion of the signal peptide with GM-CSF increased the production level from 0.8 to 40 μg/ml (Bhattacharya et al. in press). An alternative strategy has been used to express GM-CSF as inclusion bodies, however, the specific activities after final refolding are typically 10% of the correctly folded protein (Ling et al. 1995). Other hosts like baculovirus (Au et al. 1996), transgenic animals (180 μg/l) (Ryoo et al. 2001) and plant cell system (783 μg/l) (Lee et al. 2002) have been used for expression; however, the hGM-CSF expression levels were typically less than 5 mg/l. In most reports of hGM-CSF expression, only the final hGM-CSF activity have been reported, and we have not come across any reports detailing the actual production levels obtained in a bioreactor.

Yeast cells have shown the maximum potential for overexpression, especially because the secretion of the heterologous protein to the extracellular medium reduces its toxic effects and also aids purification. However, even in Saccharomyces cerevisiae, the hGM-CSF expression has been problematic with poor yields (Miyajima et al. 1986). The best results were obtained with Pichia pastoris, where the inducible alcohol oxidase I (AOX1) promoter gave a yield of 15 mg/l, whereas the constitutive GAP promoter increased the yield marginally to 20 mg/l (Wu et al. 2003). Interestingly, there are lots of reports on murine GM-CSF expression with a maximum productivity of 3.5 mg l⁻¹ h⁻¹ in S. cerevisiae (Yang and shu 1996). A problem with
Abstract Various host–vector combinations were tested to maximize the extracellular production of recombinant asparaginase in Escherichia coli. Expression of recombinant asparaginase fused to pelB leader sequence under the inducible T7lac promoter in BLR (DE3) host cells resulted in optimum extracellular production in shake-flasks. Fed-batch studies were carried out using this recombinant strain and an exponential feeding strategy was used to maintain a specific growth rate of 0.3 h⁻¹. To check the effect of the time of induction on expression, cultures were induced with 1 mM isopropyl-β-D-thiogalactopyranoside at varying cell optical densities (OD₆₀₀: 33, 60, 90, 135). Although the specific product formation rates declined with increasing OD of induction, a maximum volumetric activity of 8.7×10⁵ units l⁻¹, corresponding to ~5.24 g l⁻¹ of recombinant asparaginase, was obtained when induction was done at an OD₆₀₀ of 90. The recombinant protein was purified directly from the culture medium, using a rapid two-step purification strategy, which resulted in a recovery of ~70% and a specific activity of ~80% of that of the native enzyme.

Introduction

Escherichia coli L-asparaginase isozyme II (L-asparagine amidohydrolase, EC 3.5.1.1) is a high-affinity periplasmic enzyme that has been in use as a therapeutic agent against acute lymphoblastic leukemia (ALL) for more than 35 years (Wriston and Yellin 1973; Gallagher et al. 1989). It is administered in combination with other drugs to induce a remission of the disease (induction therapy); and at a later stage continued application of asparaginase helps in preventing further outbreaks (maintenance therapy; Muller and Boos 1998). The cure rate has now improved to 80% in children and 30–40% in adults by optimization of the use of existing drugs (Pui et al. 2002). Apart from ALL, asparaginase is also used in the treatment of Hodgkin’s disease, acute myelocytic leukemia, acute myelomonocytic leukemia, chronic lymphocytic leukemia, lymphosarcoma, reticulosarcoma and melanosarcoma (Stecher et al. 1999).

The E. coli L-asparaginase II protein is composed of four identical subunits and has a molecular mass of 141 kDa, each monomer consisting of 326 amino acid residues. This homo-tetramer can be considered as a dimer of intimate dimers and although the dimers contain all the structural elements and functional groups to create a complete active site environment, the active enzyme is always a tetramer (Swain et al. 1993).

E. coli is the most commonly used host for expressing recombinant proteins at the shake-flask level and more so for large-scale protein production in bioreactors (Lee 1996; Baneyx 1999). In E. coli, the recombinant protein is produced either in soluble form or in the form of insoluble protein aggregates, popularly called inclusion bodies. Soluble proteins can be localized in the cytoplasm or periplasm, from where in some cases it “leaks” into the extracellular medium (Cornelis 2000; Shokri et al. 2002). Although this mechanism of secretion of some over-expressed proteins is not fully known, it is assumed that it has to do with the selective passage of the protein through the outer membrane (Shokri et al. 2003).

Extracellular production of recombinant proteins in E. coli remains problematic, the major obstacles being the incomplete processing of the signal sequence and the variable secretion efficiency of the protein(s) of interest, leading to very low amounts of secretion into the medium. The formation of inclusion bodies in the cytosol or periplasm (especially when recombinant proteins are over-expressed from strong promoters), the incorrect formation of disulfide bonds during folding and an overall limited processing efficiency of the Sec and other secretion pathways also reduce secretion efficiency (Makrides 1996). The efficiency of protein secretion also depends upon the genetic properties of the host strain, the expression vector, the signal peptide, the nature of the protein being secreted.