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Pilot-scale fermentation, purification, and characterization of recombinant human Oncostatin M in *Pichia pastoris*

Ning Kong [a], Xupeng Mu [a], Hongzhi Han [b,c], Weiqun Yan [a,*]

[a] *Department of Biological Engineering, College of Pharmacy, Jilin University, 1163 Xin Min Street, Changchun 130021, PR China*
[b] *School of Public Health, Jilin University, 1163 Xin Min Street, Changchun 130021, PR China*
[c] *Journal of Jilin University Medicine Edition, Jilin University, 828 Xin Min Street, Changchun 130021, PR China*

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ABSTRACT

Oncostatin M (OSM) is a multifunctional cellular regulator that belongs to the IL-6 subfamily and can act on a wide variety of cells, which has potential roles in the regulation of gene activation, cell survival, proliferation and differentiation. In order to achieve the higher level yield of recombinant human Oncostatin M (rhOSM), we determined the optimal pH condition of rhOSM expressed in the methylotrophic yeast *Pichia pastoris* X-33 and carried out the fermentation culture of rhOSM in 80 L fermentor in a fed-batch mode. SDS–PAGE and Western blotting assays demonstrated that rhOSM was successfully expressed and secreted into the culture medium with an apparent molecular weight of 28 kDa. N-terminals were correctly processed through amino-terminal sequencing. The maximum yield of rhOSM was 280 mg/L. rhOSM was purified by phenyl Sepharose hydrophobic interaction chromatography and SP Sepharose Fast Flow cation exchange chromatography, which resulted in a final yield of purified rhOSM of 6.94 g with a recovery of 62% and a purity of 95%. The purified rhOSM had a specific growth inhibition activity of 6.26×10^4 RU/μg, which was commensurate with typical values (6.2×10^4 RU/μg) obtained with standard hOSM.

Introduction

Oncostatin M (OSM)[1] is a multifunctional cytokine of Mr approximately 28,000 that was originally isolated from conditioned media of phorbol 12-myristate 13-acetate-treated U937 histiocytic leukemia cells that had been induced to differentiate into macrophage-like cells [1]. OSM belongs to the IL-6 subfamily which comprises IL-6, IL-11, LIF, OSM, CNTF, CT-1 and CLC. The main property of the family is function redundancy [2,3]. Among the family members, OSM is most closely related to LIF structurally, functionally and genetically [4]. OSM has pleiotropic effects on many different cell types. OSM can activate target genes involved in differentiation, survival, apoptosis and proliferation [5]. OSM is involved in the regulation of the acute-phase response to injury and infection. Besides its functions in inflammation [6] and haematopoiesis [7], OSM also plays a crucial role in liver, embryonal development [8], and the immune response [9]. Dysregulation of IL-6-type cytokine signaling contributes to the onset and maintenance of several diseases, such as rheumatoid arthritis, inflammatory bowel disease, osteoporosis, multiple sclerosis and various types of cancer (e.g. multiple myeloma and prostate cancer).

The level of OSM in human plasma and serum is 24 pg/mL through a sensitive and specific enzyme immunoassay. It is almost impossible to obtain sufficient OSM from human tissue due to its extremely low quantity. Therefore, it is essential to prepare OSM through genetic engineering for large-scale production of hOSM for basic research and other applications. In previous studies, rhOSM produced by *Escherichia coli* [10], CHO cells [11] and recombinant adenovirus vector [12] has been reported. However, the low yield or high culture expense limits the use of these expression systems. *Pichia pastoris* is a eukaryotic expression system for high level and high efficiency production of recombinant heterologous proteins at relatively low cost [13]. We have reported human OSM cDNA was cloned and expressed in *Pichia* strain X-33 which phenotype is Mut$^+$ (methanol utilization plus) in shake-flask scale [14]. Here, we attempted to carry out a fed-batch culture of rhOSM in an 80 L fermentor, with the aim of allowing the production of the higher amount of the interesting protein. The purification and preliminary characterization of rhOSM were also presented in this paper.

Materials and methods

Materials

Protein markers were purchased from Takara (Dalian, China). Phenyl Sepharose, SP Sepharose Fast Flow were from Amersham Biosciences (Sweden). OSM (sc-129) polyclonal antibody was

* Corresponding author. Fax: +86 431 85650627.
E-mail address: wq_yan2008@163.com (W. Yan).

[1] *Abbreviations used:* OSM, oncostatin M; IL-6, interleukin-6; IL-11, interleukin-11; LIF, leukaemia inhibitory factor; CNTF, ciliary neurotrophic factor; CT-1, cardiotrophin-1; CLC, cardiotrophin-like cytokine; CHO, Chinese hamster ovary; DO, dissolved oxygen.

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obtained from Santa Cruz Biotechnology (USA). The standard human oncostatin M (295-OM) was purchased from R&D Systems (USA).

Human melanoma A375 cells were provided by the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Science.

Yeast culture media

Pichia pastoris was grown in YPD (1% yeast extract, 2% peptone, and 2% dextrose) or BMGY (0.1 M potassium phosphate, 1% yeast extract, 2% peptone, 1.34% YNB, and 1% glycerol, pH 6.0). BMMY was used for protein induction (0.5% methanol is added in the place of glycerol, pH 6.0). YPD–Zeocin plates (1% yeast extract, 2% peptone, 2% dextrose, 2% agar and 0.1–0.2 mg/mL Zeocin) were used for selecting multicopy transformants.

Optimum pH for expression of rhOSM

In order to achieve the higher yield of rhOSM, experimentation with pH values varied from 3.0 to 6.5 with 0.5 pH unit intervals between tubes was tested to determine the optimal pH for rhOSM. We have selected the high-level expression *P. pastoris* strains of rhOSM under the condition of pH 6.0 before [14]. The inoculum seed of a single *P. pastoris* colony of the selected strains was initially inoculated into a 500 mL shake flask containing 100 mL BMGY medium at 30 °C and 250 rpm. Until optical density at 600 nm (OD_{600}) reached 2.0–6.0 (spectrophotometer Ultrospec 3000, Amersham bioscience, Freiburg, Germany), the culture medium was divided into 10 portions and the cells were harvested by centrifugation and resuspended by 10 ml BMMY medium of different pH values in 50 ml conical tubes to induce expression. The cells were allowed to grow for 72 h at 30 °C, and methanol was added every 24 h to a final concentration of 0.5% (v/v) for induced expression of the target protein. For recombinant protein detection, 0.2 mL cell aliquots were withdrawn and the supernatant samples were run on a 12% (w/v) polyacrylamide gel and stained with Coomassie brilliant blue R250. Gel densitometry was used to quantify the proportion of rhOSM among the total proteins secreted by *P. pastoris*.

Pilot-scale fermentation culture of rhOSM

It is important to monitor and control the following parameters throughout the fermentation process: temperature; DO; pH; agitation; aeration; antifoam; carbon source. Fermentation process was divided into three phases designated glycerol batch phase, glycerol-fed batch phase, and methanol-fed batch phase.

A stock culture of *P. pastoris* was grown to an OD_{600} = 2.0–6.0 in a 5 L shake flask containing 2 L YPD. The shake-flask culture was used to inoculate an 80 L NBS Bioflo 5000 fermenter (New Brunswick Scientific, USA) containing 40 L of fermentation basal salts medium FM21 supplemented with PTM_1 trace salts (1.1 mL of stock solution/L, per liter: 6.0 g $CuSO_4{\cdot}5H_2O$, 0.08 g NaI, 3.0 g $MnSO_4{\cdot}H_2O$, 0.2 g $NaMoO_4{\cdot}2H_2O$, 0.02 g H_3BO_3, 0.5 g $CoCl_2$, 20.0 g $ZnCl_2$, 65.0 g $FeSO_4{\cdot}7H_2O$, 5.0 mL H_2SO_4) [15] and biotin (0.4 mL of the stock solution/L). The DO level was maintained at 20–30% saturation using 100% oxygen and a stirring speed of up to 700 rpm. The pH of the medium was maintained 5.0 by automatic addition of 5 M NH_4OH and 1 M phosphoric acid and 5% antifoam was delivered as required. Temperature was controlled at 28 °C. Calibrated peristaltic pumps were used to control methanol feed rate which was determined by the metabolic rate of the culture and the yield of the target protein and a methanol probe and sensor unit was employed to maintain constant levels of methanol during the induction phase.

At the end of the first phase, when glycerol was consumed, as indicated by a sharp increase in the DO concentration, the second phase was initiated. A 50% glycerol feed, containing 1.2% (v/v) of PTM_1 trace salts, was added at 400 mL/h and gradually increased to 720 mL/h. The glycerol feed was carried out until a cell yield of 180–220 g/L wet weight was achieved. The third phase was initiated by starting a 100% methanol feed containing 1.2% (v/v) of PTM_1 trace salts. During the methanol induction phase, pH was increased to 5.5. Methanol was initially added at 144 mL/h for 4 h to adapt the culture to growth on methanol, then gradually increased to 440 mL/h. Sampling of the culture medium was performed at the end of each phase and at least twice daily and analyzed for wet cell weight, optical density at 600 nm and expression level of rhOSM.

Purification of rhOSM

The fermentation broth was harvested from bioreactor at the optimal time points of methanol induction phase and centrifuged to separate the supernatant from the cells at 12,000 rpm for 15 min. The supernatant was added into ammonium sulphate to reach about 25% (g/L) and adjusted to pH 7.0 with 5 M NH_4OH and then stored at 4 °C for 5 h. After 5 h, the supernatant was centrifuged again (4 °C 12,000 rpm for 20 min). 200 mmol/L sodium phosphate buffer (pH 7.0) of 1/10 total volume was added to the supernatant and the supernatant was loaded onto a phenyl Sepharose column which was equilibrated with 25% ammonium sulphate in 20 mmol/L sodium phosphate buffer (pH 7.0). The column was washed extensively with the same buffer. The bound protein was eluted with a linear gradient of 25–0% ammonium sulphate (in 20 mmol/L sodium phosphate buffer, pH 7.0). Aliquots were collected from the various fractions across the major peak and analyzed by SDS–PAGE, western blotting and Bradford protein assay to identify where the product elutes. The elution fractions containing rhOSM were pooled and diluted to reach suitable ionic strength and loaded onto a SP Sepharose Fast Flow column equilibrated with 20 mmol/L NaAc–HAc (pH 4.5) buffer for the further purification. The column was eluted with a linear salt gradient (0–0.5 M NaCl) and the protein was monitored by measuring the UV absorbency at 280 nm. The pooled elution fractions containing rhOSM from SP Sepharose Fast Flow column were dialyzed and filtrated on 0.22 μm filter. The purified rhOSM was carried out on a HPLC system (Waters 600E, USA) using a C18 reverse phase column for purity analysis. The final purified rhOSM solution was stored under sterile conditions at −80 °C for detection of bioactivities.

Protein assay

The protein concentrations in the samples were determined with Bradford protein assay [16] using bovine serum albumin as the concentration standard.

SDS–PAGE and Western blotting assays

Samples during purification were analyzed by SDS–PAGE performed using a 12% gel and stained with Coomassie brilliant blue, as described by [17]. For Western blotting [18], proteins in the gel were transferred to a polyvinylidene difluoride membrane using a semi-dry electroblotting apparatus (Bio-Rad) at 15 V for 30 min in 25 mM Tris–192 mM glycine. The membrane was blocked by incubating with solution containing 5% BSA for 1 h, and then incubated with the rabbit anti-human OSM polyclonal antibody. After being washed, the membrane was incubated with the goat anti-rabbit IgG conjugated to HRP (Dingguo, China). The bound antibody was detected using 3,3'-diaminobenzidine (DAB).