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High level expression, efficient purification and bioactivity assay of recombinant human platelet-derived growth factor AA dimer (PDGF-AA) from methylotrophic yeast *Pichia pastoris*

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ABSTRACT

Platelet-derived growth factors (PDGFs) are important biochemical mediators regulating many physiological and pathophysiological processes, including promotion of the chemotactic recruitment and proliferation of cells involved in wound repair. Previously, homodimers of rhPDGF-AA protein were purified from *Escherichia coli*. However, eukaryotic proteins often contain posttranslational modifications, such as glycosylation, that are required for biological functions. In this study, an efficient method was established to purify a glycosylated rhPDGF-AA dimer from *P. pastoris* culture media by one step CM Sepharose ion exchange chromatography yielding about 20 mg/L of over 95% highly purified rhPDGF-AA. Mass spectrometry analysis of the purified rhPDGF-AA displayed a molecular weight (MW) of 27,825.513 Da, composed of a subunit with MW of 15,042.945 Da and a subunit with MW of 12,904.374 Da. The size difference is accounted for by differential glycosylation of the monomers. Biological activity of the rhPDGF-AA was confirmed by its ability to induce NIH/3T3 cells proliferation. The experimental procedure we have developed facilitates production of an active glycosylated rhPDGF-AA in large amounts for further research and drug development.

Introduction

The platelet-derived growth factors (PDGFs)[1] are major mitogens for fibroblasts, smooth muscle cells, and several other cell types, mainly of mesenchymal origin [1]. So far, four members of PDGF family have been identified, i.e. the classical PDGF-A and PDGF-B that were discovered about three decades ago, and two novel PDGFs, PDGF-C and PDGF-D, which were discovered two decades later [2–4]. PDGFs consist of five different forms of disulphide-linked dimers built up from the above four distinct polypeptide chains, including PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC and PDGF-DD [5,6]. All PDGFs contain the growth factor domain, N-and C-terminal polypeptide sequences that are essential for the biological activities of these factors [7,8].

PDGF-AA is a key regulator in directional cell migration during embryonic development and a chemoattractant during postnatal migratory responses including wound healing [9]. PDGF-AA was observed in capillaries and fibroblasts of acute wounds. Multiple transcripts of PDGF-AA have been detected. They appear to be alternative splice variants of a single seven exon gene, and give rise to short (S) and long (L) processed proteins of 110 (A_S) and 125 (A_L) amino acids. During the early healing process, the long form of PDGF-AA is more prevalent while the short form of PDGF-AA, which is more efficiently diffusible, becomes prevalent at the later phase [10,11]. The gene of human PDGF-A was located on chromosome 7, exons 4 and 5 encode most of the mature protein [12,13]. The mature PDGF-AA is a positively charged (pI = 9.8–10) hydrophilic protein. The mature native PDGF isoforms migrate in SDS-gel electrophoresis as components of 30 kDa, whereas the reduced chains migrate as 15-kDa components [14,15].

Previously, homodimers of rhPDGF-AA protein were purified from *Escherichia coli*. In this study, we expressed rhPDGF-AA using the yeast *P. pastoris* and report our establishment of a highly effi-

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[1] *Abbreviations used:* PDGFs, platelet-derived growth factors; BMMY, buffered methanol complex medium; YNB, yeast nitrogen base; NMWL, nominal molecular weight limit.

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cient purification procedure for a glycosylated and biologically active rhPDGF-AA in *P. pastoris*.

Materials and methods

Strains, plasmids, media and reagents

P. pastoris strain X-33, expression vector pPICZαA, yeast nitrogen base (YNB), D-sorbitol, D-biotin, and Quant-iT™ Protein Assay kit were purchased from Invitrogen (CA, USA). Plasmid extract kit, restriction enzymes, DNA polymerase and T4 DNA ligase were purchased from Takara (Guangzhou, China). Primers 5′ Factor and 3′ AOX_1 was synthesized by Invitrogen (CA, USA). *E. coli* transformants were selected on LB agar plates containing low salt (1% peptone, 0.5% NaCl, 0.5% yeast extract, and 1.5% agar containing 25 mg/L zeocin. *P. pastoris* transformants were initially selected on YPDS plates (1% yeast extract, 2% peptone, 2% dextrose, 1 M sorbitol, 2% agar, and 100 mg/L zeocin), then YPD (same as YPDS except that sorbitol was omitted) plates containing 500 mg/L zeocin were used for the isolation of single colonies. Amicon Ultra centrifugal filter devices-3000 nominal molecular weight limit (NMWL: 3 kDa) were purchased from Millipore (Guangzhou, China). AKTA FPLC and CM-Sepharose FF ion exchange columns (Hi Trap™, 1 ml) were purchased from GE Health care (Guangzhou, China). Trifluoromethanesulfonic acid was purchased from Sigma (Guangzhou, China). All other reagents used were of reagent grade (Sigma, Guangzhou, China).

Plasmid construction

Human PDGF-A cDNA (330 bp) was amplified by PCR from a plasmid encoding mature human PDGF-A transcript variant 2 cDNA (NCBI Reference Sequence, NM_033023.4). An *Xho*I site was introduced to allow in-frame cloning into α-factor secretion signal of pPICZαA and a nucleotide sequence encoding the KEX2 cleavage site was placed upstream of the PDGF-A. Forward and reverse primers used were 5′-GCCTCGAGAAAAGAAGCATCGAGGAAGCTGTCC-3′ and 5′-GCTCTAGA TTACCTCACATCCGTGTCCTC-3′, respectively. The PCR products were digested with *Xho*I and *Xba*I, the digested fragments were inserted between the *Xho*I and *Xba*I sites of pPICZαA where the PDGF-A is under the control of alcohol oxidase1 promoter in this construct.

Transformation of P. pastoris and selection of transformants

The expression vector was linearized by digestion with *Sac*I and transformed into *P. pastoris* X-33 using electroporation as recommended (Invitrogen, 2008). *P. pastoris* transformants were plated on yeast extract peptone dextrose (YPDS) plates containing 100 μg/ml of zeocin. Resulting colonies were transferred to YPD plates containing 500 μg/ml of zeocin. After incubation at 30 °C for 72 h, large colonies were picked and subjected to PCR amplification using primers 5′ factor and 3′ AOX1. The confirmed transformants were selected for further studies.

Small-scale fermentation and time course expression study

Selected colonies from the YPD plate containing 500 μg/ml of zeocin were grown in 250 ml BMGY growth medium in 1 L flask with constant shaking (250 rpm) at 30 °C for 18 h till the OD_{600} measured between 8–10. The cultures were centrifuged for 5 min at 2000×*g* and pellets were collected. For the induction phase, the collected cell pellets were inoculated into 50 ml of BMMY (buffered methanol complex medium) induction medium in 500 ml baffled flask and grown for 5 days at 28 °C with constant shaking at 250 rpm, as recommended by the manufacturer (Invitrogen, 2008). Each day, 1 ml of 100% methanol was added per liter of culture (final concentration of 1.0%) to induce the protein expression. One ml cell culture was collected at 0 h, 12 h, 24 h, 48 h, 72 h, 96 h and 120 h after the addition of methanol and centrifuged at 16,000×*g* for 1 min at room temperature. 0.2 ml of the supernatant was mixed with 0.6 ml of acetone. After centrifugation, precipitates were re-suspended in 50 μl of 1× SDS reduced loading buffer. The samples were subjected to SDS–PAGE (15% gel) analysis.

Expression and purification of rhPDGF-AA

P. pastoris harboring pPICZαA/*hPDGF* with resistance to 500 μg/ml zeocin was grown in 2 bottles of 0.6 L BMGY medium by constant shaking (250 rpm) at 30 °C till OD_{600} = 8–10. Cells were harvested and re-suspended in two 3 L baffled flask containing 0.12 L BMMY medium (contains 1.0% methanol), then cultured for 96 h. Methanol was added to the medium to a final concentration of 1.0% every 24 h. After the 96 h induction, cell cultures were centrifuged for 15 min at 20,000×*g*. 200 ml supernatant was collected and dialyzed against 3 L buffer A (20 mM Tris–HCl, pH 8.0) at 4 °C overnight. After centrifugation at 20,000×*g* for 30 min at 4 °C, the dialyzed supernatants against buffer were loaded onto a respective 1 ml CM Sepharose column which was pre-equilibrated using buffer A. Then the columns were washed with 200 ml buffer B (20 mM Tris–HCl, 50 mM Nacl, pH 8.0) to remove unbounded proteins. The recombinant rhPDGF-AA was eluted with buffer C (20 mM Tris–HCl, 150 mM Nacl, pH 8.0), and the eluted fractions were subjected to SDS–PAGE analyze and stained with Coomassie blue R250.

Matrix assisted laser desorption ionization-mass spectroscopy

A PerSeptive Biosystem's matrix assisted laser desorption ionization Voyager-DE MALDI TOF mass spectrometer (Framingham, MA/Applied Biosystems, Foster City, USA) equipped with a 337 nm nitrogen laser was used. The rhPDGF-AA and reduced rhPDGF-A protein (by DTT) were dissolved and desalted by dialysis against water. The protein solution was diluted (5 pmol/μl) in matrix solution (alpha-4-hydroxycinnamic acid dissolved in 50% aqueous acetonitrile containing 0.1% TFA). All MALDI spectra were calibrated externally and the determination of molecular mass was conducted with about 50 scans averaged for each analysis.

Deglycosylation with TFMS

Trifluoromethanesulphonic acid (TFMS) is very effective at removing O- and N-linked oligosaccharides. Deglycosylaion of rhPDGF-AA was performed in a moisture-free atmosphere using anhydrous TFMS [16]. 50 μl of anhydrous TFMS (Sigma) was added into sample of moisture-free rhPDGF-AA and incubated at −10 °C for 15 min. The reaction was terminated by adding 800 μl of 2 M Tris–base (Bio-Rad). The PDGF-AA sample was dialyzed in a dialysis bag against 1 L ice-cold dialysis buffer I (20 mM Tris–HCl, pH 8.5) for overnight. The deglycosylated PDGF-AA was concentrated in 15 ml ultracentrifuge filters (Millipore, USA) with molecular weight cut-off (MWCO) of 3 kDa and analyzed by 12% SDS–PAGE.

Cell proliferation assay

The assay was conducted in a 96-well plate format. NIH/3T3 cells were cultured in DMEM containing 10% (v/v) FBS (fetal bovine serum) in a 10 cm cell culture dish. On day 2 when the cells were 80–90% confluent, cells were digested by 2 ml Trypsin–EDTA solution (0.05% Trypsin and 0.02% EDTA) until the cells became rounded in shape. After removal of the Trypsin–EDTA solution,