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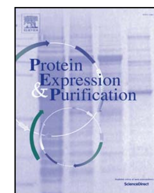

Ligand-free method to produce the anti-angiogenic recombinant Galectin-3 carbohydrate recognition domain

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ABSTRACT

Galectin-3 (Gal3) is involved in many physiological processes related to tumor growth, such as promoting angiogenesis, cell migration/invasion, resistance to apoptosis and immune response modulation. Usually the overexpression of Gal3 is a poor prognostic marker for cancer patients. Recombinant Gal3 carbohydrate domain (Gal3C) has been proposed as a useful tool to inhibit angiogenesis. So far, all production protocols reported for Gal3C production have used proteolytic cleavage of full length Gal3 and/or affinity-based purification. This involves dialysis, a time consuming step used to eliminate the elution ligand, usually lactose. In this report, we describe an alternative method to produce human recombinant Gal3C in *E. coli*, purified with cationic exchange and size exclusion chromatography. The recombinant protein was characterized using circular dichroism and nuclear magnetic resonance, showing a beta sheet enriched well-folded globular structure. The average yield obtained was 26 mg/L of broth and the purity was above 99%. The anti-angiogenic activity was assessed *in vitro* and showed a reduction of 70% and 77% in endothelial cells tubule formation upon treatment with 10 and 20 μg/mL, respectively and also had no impact on cell viability. The method described here is more suitable for both laboratory and industrial production of the potential anti-tumor Gal3C.

1. Introduction

Tumor growth depends on many factors such as constant proliferative signaling, ability to evade tumor suppressors, invasiveness and metastasis formation, resistance to cell death, and induction of angiogenesis [1,2]. More recently metabolism deregulation, immune system evasion, inflammation promotion and genomic instability were added, and taken together are considered the hallmarks of cancer [3].

An inflammatory microenvironment plays central roles in the development and growth of many solid tumors because it supplies the tumor and adjacent cells with growth and survival factors, extracellular matrix modifying enzymes and angiogenic factors such as VEGF (Vascular endothelial growth factor) and galectin-3 (Gal3) [4]. Release of VEGF by a tumor and immune cells recruits myeloid derived circulating cells, which differentiate into Tumor associated macrophages (TAMs) and further enhance the production of VEGF [5].

The presence of Gal3 in tumors is associated with poor prognosis and levels of circulating Gal3 are higher in cancer patients. This protein is involved in angiogenesis promotion, in cell migration and invasion, apoptosis resistance and immune response modulation [2,6–8].

Human Gal3 is composed of 250 amino acid residues corresponding to approximately 35 kDa Gal3 has two protein domains, an N-terminal domain required for oligomerization, and a C-terminal carbohydrate binding domain (CRD) that binds preferentially to glycoconjugates containing β-galactosides. Gal3 can also form homodimers via CRD interaction, abolishing its carbohydrate binding function [9,10].

Secreted Gal3 participates in cell/extracellular matrix interaction, impacting metastasis development and angiogenesis in solid tumors, suggesting that Gal3-mediated cellular processes are putative targets to anti-cancer therapy [8,11]. Previous studies have shown that treatment of ovarian tumor cells *in vitro* with a truncated form of Gal3 CRD (Gal3C) was able to reduce cell survival, invasion and migration [8]. In addition, treatment with Gal3C also impaired the tubulogenic capacity of endothelial cells *in vitro* [7,12]. *In vivo* mouse models of multiple myeloma and breast tumors showed that Gal3C treatment combined with chemotherapeutic agents had a greater response than

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chemotherapy alone [13–15].

Current protocols to produce and purify recombinant Gal3C require expression of full-length galectin-3, followed by affinity purification on lactose resin and proteolytic cleavage generating the desired protein fragment. This is followed by another step of chromatographic purification to isolate Gal3C [13,14]. In addition, Duckworth et al. reported an affinity tag-fused Gal3C production [12].

In this work, we describe the expression and purification of an untagged Gal3C, using a less-expensive and more straightforward two-step chromatographic routine. Using this method we were able to produce high yields of recombinant protein free of carbohydrate ligands. Furthermore, purified recombinant Gal3C inhibited *in vitro* tubule formation of endothelial cells, without impact on cell viability.

2. Material and methods

2.1. Reagents and materials

Triptone, agar and plastic material for cell culture were obtained from KASVI (Curitiba, Brazil), isopropyl b-D-1-thiogalactopyranoside (IPTG) from Uniscience (São Paulo, Brazil). Sodium ampicillin was obtained from INLAB (São Paulo, Brazil). Sodium phosphate monobasic, Sodium phosphate dibasic, sodium chloride, sodium hydroxide, yeast extract, protease inhibitor SigmaFAST EDTA-Free, cell culture antibiotics (Pen/Strep) and urea were obtained from Sigma-Aldrich (St. Louis, MO), DMEM Medium was from Gibco (Grand Island, NY). Fetal bovine serum was from LCG Biotechnology (São Paulo, Brazil), and Matrigel® from Corning Life Sciences (Salt Lake *City*, UT). HiTrap SP HP and 16/60 Superdex 75 columns were from GE Healthcare (Little Chalfont, UK). 3 kDa Amicon concentrators are from Millipore (Billerica, MA). pET-25b(+) plasmid containing the optimized cDNA for Gal3C was synthetized by GenScript USA Inc. (Piscataway, NJ). ACTGene Protein Marker was obtained from ACTGene (Piscataway, NJ).

2.2. Gal3C overexpression and purification

The coding sequence of Gal3C (Uniprot: P17931), comprising amino acids 103 to 250 optimized to *Escherichia coli* expression, was cloned into pET25b(+) plasmid using restriction sites *Nde*I and *EcoR*I. The construction was transformed into E. *coli* BL21(DE3) (SigmaAldrich, St. Louis, MO) and the cells were grown in 300 mL of Luria-Bertani (LB) medium with ampicilin 100 μg/mL at 37 °C. When cultures reached OD_{600} values of 0.7, IPTG was added at a final concentration of 1 mM. Cells were incubated for 6 h under 220 rpm agitation at 37 °C. After that, cells were harvested by centrifugation at $8000 \times g$ for 5 min and resuspended in 10 mL of Buffer A (20 mM Sodium Phosphate pH 7.4) and protease inhibitor. Cells were disrupted by intermittent ultrasonication 12–16 Ω, 60 min, 5/5 s (Microson® Ultrasonic Cell Disruptor, Qsonica, LLC, Newtown, CT) on an ice bath and the lysate was separated by centrifugation at $6000 \times g$ for 30 min at 4 °C. The supernatant was loaded into two in tandem HiTrap SP HP 5 mL columns pre equilibrated with Buffer A. The resin was then washed with 10 column volumes of buffer A and the bound proteins were eluted with 120 mL of a linear gradient of 0–100% Buffer B (20 mM Sodium Phosphate, 1 M NaCl pH 7.4). Fractions of interest were concentrated by ultrafiltration with 3 kDa molecular weight cut-off Amicon membranes. The concentrated samples were loaded on Superdex 75 16/60 size exclusion chromatography column pre-equilibrated with Buffer C (20 mM Sodium Phosphate, 150 mM NaCl pH 7.4). Final sample concentration was determined by measurement of absorbance at 280 nm (Biospectro SP-220, Curitiba, Brasil) considering a molar extinction coefficient of 11460 $M^{-1}cm^{-1}$ (Expasy Protparam Tool available in http://web.expasy.org/protparam). Purified Gal3C was stored at 4 °C until subsequent biophysical characterization and activity assays. Protein expression and purification was monitored by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [16]. A SRT SEC-150 Sepax column (5 μm; 150 Å; 7.8 × 300 mm) was also used to determine the purity of Gal3C in 20 mM sodium phosphate buffer, at pH 7.4. Column was run at 1 mL/min and monitored by OD_{280nm} and $OD_{214\ nm}$. The following globular proteins were used as standards: Bovine Serum Albumin – dimer 132 kDa and monomer 66 kDa (Sigma-Aldrich, A7906); Carbonic Anhydrase – 21 kDa (Sigma-Aldrich, C7025); Cytochrome C – 12 kDa (Sigma-Aldrich, C2506). Results were analyzed using the software GraphPad Prism v.6.

2.3. Gal3C structure analysis

2.3.1. Circular dichroism (CD) spectroscopy

Circular dichroism (CD) measurements were performed on a spectropolarimeter at 25 °C (J-715, Jasco Inc., Easton, MD) in a quartz cell of 0.1 cm path length, with an average over three scans per spectrum at a speed of 0.5 nm/min. CD spectra were recorded using 20 μM Gal3C in 5 mM sodium phosphate buffer and 37.5 mM NaCl, pH 7.4, to minimize CD signal disturbance, and in the absence or in the presence of 7 M urea. Far-UV spectra were collected from 260 to 190 nm, in steps of 0.5 nm. The buffer baselines were subtracted from the respective sample spectra. Two CD spectra were recorded in different days with the same setup. Secondary structure content was calculated with K2D3 software available at http://k2d3.ogic.ca.

2.3.2. Nuclear magnetic resonance (NMR) spectroscopy

NMR experiments were performed with using Gal3C at 200 μM in 20 mM sodium phosphate buffer, at pH 7.4, 150 mM sodium chloride, and 10% deuterium oxide. 1D ^{1}H NMR spectrum was acquired on an 800 MHz spectrometer (Bruker Avance III, Bruker Billerica, MA), using a z-axis gradient 5 mm triple resonance probe at 25 °C, with a total of 1024 scans, and processed with Topspin 3.5 (Bruker).

2.4. Tubule formation assay

Matrigel® aliquots were placed in 96-well plates (50 μl/well) and polymerized for 1 h at 37 °C. EA.Hy926 cells (ATCC CRL-2922) were plated in the coated wells (2×10^4 cells) in 200 μL of DMEM medium supplemented with 10% FBS, and treated with Buffer C (negative control), 0.1 M lactose (positive control), and 10 or 20 μg/mL Gal3C. Cells were incubated in a 5% CO_2 humidified atmosphere at 37 °C for 18 h. Cell growth and tridimensional organization (Cells exhibiting branching points) were observed and visually quantified using an inverted optical microscope [17].

2.5. Viability assay

EA.Hy926 cells were seeded in 96-well plates (5×10^3 cells/well) and allowed to attach for 24 h and then treated with Buffer C (negative control), 0.1 M lactose (positive control), and 10 or 20 μg/mL Gal3C in DMEM medium supplemented with 10% FBS for 48 h. Cellular viability was assessed using (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma Co., M5655), as previously described [18].

2.6. Statistical analysis

All activity data were expressed as mean ± SD. One-way ANOVA followed by Bonferroni's multiple comparisons was used to compare the different treatments to control condition. All analyses were performed using GraphPad Prism version 6.05 (GraphPad Software, La Jolla, CA).

3. Results and discussion

3.1. Overexpression and purification of Gal3C

To the best of our knowledge, the established methods for

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