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One-step FPLC-size-exclusion chromatography procedure for purification of rDMBT1 6 kb with increased biological activity

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ABSTRACT

Deleted in Malignant Brain Tumor 1 (DMBT1, alias SAG or gp340) is a pattern recognition receptor involved in immune defense, cell polarization, differentiation and regeneration. To investigate the role of the protein in physiological and pathological processes, the protein has often been isolated from saliva or produced *in vitro* and purified by a multistep affinity purification procedure using bacteria, followed by FPLC. Here, we compared a simple, one-step FPLC-SEC protocol for purification of recombinant DMBT1 6 kb, with that of the standard bacteria affinity purification-based protocol. Our data suggest that our FPLC-SEC protocol yields DMBT1 in a more native conformation.

Introduction

The DMBT1 protein (Deleted in Malignant Brain Tumor 1), also referred to as glycoprotein-340 (gp340), and salivary agglutinin (SAG), is encoded by the DMBT1 gene localized at chromosome 10q25.3-q26.1 and identified as a putative tumor suppressor in malignant gliomas [1]. The multi-functional protein DMBT1 is mainly expressed by epithelial cells such as the lung and the small intestine, and plays a role in mucosal immune defense [2]. Further, it is expressed by different immune cells and linked to innate host defense, but is also present in the extracellular matrix (ECM), where it is likely involved in processes related to cell polarization and differentiation as well as regenerative processes such as wound healing [3].

Because of its bacteria recognition properties, which have been shown to be “pattern-mediated”, the protein is considered a pattern recognition molecule (PRM) or pattern recognition receptor (PRR), an ancient family of receptors associated with the innate immune response [4]. Moreover, thanks to its pattern recognition properties, synthetic peptides from the protein, have been used to design nanoparticles for siRNA delivery [5]. The size of the DMBT1 monomer is about 300 kDa, but the protein undergoes oligomerization and glycosylation leading to high molecular weight oligomers (~6000 kDa) before being secreted to the extracellular environment [6]. Moreover, DMBT1 oligomers have been found to self-assemble after secretion, giving rise to large aggregates. Among the existing species, only the oligomers and the aggregates are biologically active [7].

Being involved in a large array of physiological and pathological processes [8], DMBT1 has been studied in many contexts, and its production and purification processes are of significant interest. While salivary DMBT1 can be obtained directly from saliva ($DMBT1_{SAG}$), or from pulmonary secretion (GP340), recombinant DMBT1 (rDMBT1) can be generated *in vitro*.

To isolate the protein either from saliva or from the cell medium in which it is secreted, two protocols have been established, one being a two- or three-step fast protein liquid chromatography (FPLC) purification [2,6], and the other a multistep affinity purification with bacteria (either S. *gordonii* or S. *mutans*), sometimes followed by size-exclusion chromatography (SEC) [9–16].

In this work we tailored a one-step FPLC-SEC purification protocol for rDMBT1 6 kb variant based on the fact that DMBT1 oligomers are expected to assemble in aggregates of larger size than other proteins in the medium, and such aggregates were, indeed, observed upon microscopy of DMBT1-expressing cells. We also compared the affinity of the rDMBT1 6 kb variant obtained by our method with that of the most commonly used bacteria affinity purification protocol and found similar yields and purity, and our more efficient FPLC-SEC-purification method yielded DMBT1 with improved affinity for S. *mutans*.

Although the FPLC-SEC is a common technique used for the

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separation of proteins, this has been used to purify the DMBT1 protein in a multistep procedure [2,6], or rarely in as a one-step procedure [17], while the most common technique is the affinity purification procedure using bacteria [9–16]. Our experiments reveal that the affinity enrichment step based on the use of S. mutans, not only is unnecessary, but also impairs the capability of DMBT1 in agglutinating bacteria.

Material and methods

Production of recombinant DMBT1 6 kb

Recombinant A549 lung cancer cells expressing rDMBT1 6 kb were used to produce the protein. The A549 cell line obtained from the American Type Culture Collection (ATCC) was genetically modified using a protocol previously reported for the insertion of human genes [18]. First, an acceptor cell line was generated via site-directed Flp-FRT recombination technology. Subsequently, the cell line was used to insert the coding sequence of the protein DMBT1 6 kb, with tetracycline-induced expression. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich) containing 10% FBS (Sigma-Aldrich), 100 U/µl Penicillin/Streptomycin (Sigma-Aldrich) and 500 µg/ml Hygromycin B (Invitrogen), and maintained at 37 °C in humidified, 5% CO2. At 80% cell confluency, the medium was replaced with DMEM containing 10% FBS, 100 U/µl penicillin/streptomycin and 10 µg/ml doxycyclin (Sigma-Aldrich) to induce rDMBT1 6 kb expression, which was then secreted by the cells into the medium. After 96 h, the supernatant containing DMBT1 was aspirated, centrifuged for 8 min at 1200 g to remove detached cells and cell debris and stored at −20 °C.

Isolation of recombinant DMBT1 6 kb

Multi-step isolation: S. mutans affinity enrichment followed by FPLC purification

S.*mutans* bacteria (kindly provided by Prof. Mogens Kilian, Aarhus University) were cultured at 37 °C under aerobic conditions in Brain Heart Infusion Broth (Sigma-Aldrich). 250 ml of bacterial medium at OD_{700} = 0.7 were isolated by centrifugation (10 min, 5000 g, 4 °C) and washed with 50 ml of 10 mM EDTA in PBS. Subsequently, the bacteria were centrifuged again (10 min, 5000 g, 4 °C) and resuspended in 20 ml of PBS at OD_{700} = 1.7. The bacterial suspension was mixed with 20 ml of DMEM containing rDMBT1 6 kb and incubated 45 min at 37 °C while shaking. The bacterial aggregates were then separated by centrifugation (10 min, 5000 g, 4 °C). To elute the protein, the bacteria were resuspended two times in 10 ml of 20 ml of 10 mM EDTA in PBS and incubated for 1 h at RT, while shaking, for a final volume of 20 ml. A final centrifugation (10 min, 5000 g, 4 °C) was performed to remove the bacteria and recover the supernatant containing rDMBT1 6 kb, which was then sterile-filtered, using 0.22 µm syringe filters (EMD Millipore, SLGP033NB) to ensurecomplete removal of bacteria or bacteria-protein aggregates. Then, 20 ml of eluate was concentrated to 1 ml using centrifugal concentrators (Vivaspin®20, 300000 MWCO PES). Subsequently, rDMBT1 6 kb was purified by FPLC-SEC using a Superose 6 10/300 GL column (GE Healthcare) equilibrated with PBS. The fractions were analyzed by polyacrylamide gel electrophoresis (Bolt®, Invitrogen, see paragraph 2.4 for details), followed by silver staining (Pierce™ Silver Stain Kit) and, as expected due to the oligomeric nature of the protein, rDMBT1 6 kb was found in the initial fractions corresponding to the column void volume. The calibration of the Superose 6 10/300 GL column using the gel filtration standard from BIO-RAD (product number 151–190) is shown in Fig. S1a, and the FPLC-SEC purification of rDMBT1 6 kb on the same column is shown in Fig. S1b. The silver staining of SDS-PAGE of selected fractions from the FPLC-SEC purification of rDMBT1 6 kb (Fig. S1c), confirms that the purified protein was in the void volume. Fractions containing the protein were pooled and stored at −20 °C.

One-step FPLC-SEC purification

DMEM containing rDMBT1 6 kb (20 ml) of the same lot used for the bacteria affinity purification was concentrated to 1 ml using centrifugal concentrators, omitting the bacterial aggregation step (Vivaspin®20, 300000 MWCO PES). Subsequently, rDMBT1 6 kb was purified by FPLC-SEC as described in paragraph 2.2.1.

Comparison of purification methods

Quantification of rDMBT1 6 kb by ELISA

The concentration of the purified protein was quantified by bicinchoninic acid assay (Pierce™ BCA Protein Assay Kit) following manufacturer's instructions. rDMBT1 6 kb concentration in the initial medium was quantified by enzyme-linked immunosorbent assay (ELISA) using High-binding 96-well plates (Greiner). Different concentrations (1–30 µg) of pure rDMBT1 6 kb were used to build a standard curve. Plates were incubated with the samples overnight at 4 °C, washed and blocked with 2% BSA in PBS overnight at 4 °C, and subsequently incubated with a mouse monoclonal anti-DMBT1h12 antibody generated in-house. The antibody recognizes a non-repetitive, non-SRCR domain, epitope located at amino acid 26–40, which is present within all known DMBT1 variants and was used in previous DMBT1-related works [19]. After 2 h incubation at 37 °C, the plate was washed and incubated with a FITC-labeled goat anti-mouse secondary antibody (AffiniPure F(ab')2 Fragment Goat Anti-Mouse IgG, Jackson ImmunoResearch) by 2 h at 37 °C. Three washes with PBS containing 0.1% Tween were performed between each step. Finally, FITC fluorescence was measured with a VICTOR3 Multilabel Plate Reader (PerkinElmer).

Evaluation of purity of rDMBT1 6 kb by silver staining

Purified protein (1 µg) were added with NuPAGE LDS Sample Buffer (Invitrogen) and NuPAGE Sample Reducing Agent (Invitrogen) to ensure denaturing and reducing conditions, and incubated at 70 °C for 15 min. Subsequently, the samples were separated on precast 3–8% linear gradient SDS-polyacrylamide gels (SDS-PAGE) (Invitrogen) using Novex Trys-Glycine SDS running buffer (Invitrogen) for 35 min at 200 V. The resulting gels were silver stained (Pierce™ Silver Stain Kit) and analyzed using the Image J gel analysis tool (http://rsbweb.nih.gov/ij/index.html) to measure density after background subtraction.

Evaluation of rDMBT1 6 kb activity by bacteria agglutination test

The bacteria agglutination assay was adapted from Ref. [9]. In brief, 100 µl of an S. *mutans* bacterial suspension (OD_{700} = 1) were mixed with 20 µl of rDMBT1 6 kb (6.5 µg/ml solution) or PBS in 96-Well Microtiter™ Microplates (Thermo Scientific™), and turbidimetry was monitored at 700 nm for 60 min with a VICTOR3 Multilabel Plate Reader (PerkinElmer).

Results and discussion

The initial concentration of DMBT1 in the cell medium was 20 µg/ml, as measured by enzyme-linked immunosorbent assay (ELISA). Two aliquots of 20 ml of the same lot were used to purify the protein using the two different methods (see Fig. 1).

The final concentration of purified protein measured by bicinchoninic acid assay (BCA) averaged to 47% for the bacteria-affinity protocol followed by FPLC-SEC, and to 56% for the single FPLC-SEC protocol (Fig. 2b).

The final purity of the protein evaluated by silver staining of SDS-PAGE (Fig. 2a) and measured by the software ImageJ was slightly higher in our one-step FPLC-SEC purification compared to the bacteria-affinity protocol (94% vs. 89%) (Fig. 2c).

Finally, the biological activity of the purified rDMBT1 6 kb by the two methods was measured by the capacity of rDMBT1 6 kb to recognize and aggregate S. *mutans* by OD_{700} decrease over time.