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J Immunol Methods. Author manuscript; available in PMC 2016 September 01.

Published in final edited form as:

J Immunol Methods. 2015 September ; 424: 106–110. doi:10.1016/j.jim.2015.05.008.

Rapid and efficient purification of ficolin-2 using a disposable CELLine bioreactor

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Abstract

The human opsonin ficolin-2 (L-ficolin) is an innate pattern-recognizing molecule that binds to acetylated moieties. Upon binding, ficolin-2 activates complement through the lectin pathway, opsonizing the target to promote phagocytic clearance. Ficolin-2 has been found to interact with a growing number of pathogenic bacteria, fungi, and viruses. Ficolin-2 also has proposed roles in host homeostasis, including the clearance of apoptotic cells. Consequently, there is an increased interest in studying ficolin-2, and access to purified ficolin-2 is necessary for these studies. Ficolin-2 purified from serum, plasma, or cell culture supernatants has been a useful tool in the characterization of ficolin-2 function; however, available protocols are laborious and inefficient, requiring additional processing of starting materials (e.g., polyethylene glycol precipitation or dialysis) and multiple steps of purification. Here, we investigated a simple solution to the problem: use of a simple, disposable bioreactor requiring only standard tissue culture equipment. Using this system, we generated cell culture supernatants containing high concentrations of recombinant ficolin-2, which permitted rapid purification of high-purity recombinant ficolin-2 without processing the supernatants. Purified recombinant ficolin-2 retained its binding capacity and supported complement activation in vitro. Bioreactor cultivation will likely be generally useful in the production of other recombinant proteins in the study of the complement system.

Keywords

complement; lectin pathway; L-ficolin; innate immunity; pattern recognition molecule; recombinant protein

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1. INTRODUCTION

Since its discovery in the late 1990s, ficolin-2 (also known as L-ficolin) has become a molecule of great interest. Ficolin-2 functions as an innate immune opsonin and participates in the lectin pathway of complement activation, similarly to mannose binding lectin (MBL) (Faro et al., 2008). As part of innate immunity, ficolin-2 has been described to interact with acetylated and other moieties on an ever-expanding list of pathogenic bacterial and viral targets (Fujieda et al., 2012; Kilpatrick and Chalmers, 2012; Pan et al., 2012; Luo et al., 2013; Brady et al., 2014a; Hamed et al., 2014; Vassal-Stermann et al., 2014), but it has also been shown to bind to apoptotic cells (Kuraya et al., 2005; Jensen et al., 2007) and mitochondria (Brinkmann et al., 2013), suggesting important roles in host homeostasis.

Unlike its biological function, the structure of ficolin-2 is well-understood. Ficolin-2 is comprised of an N-terminal cysteine rich domain, a long, collagen-like domain, and a C-terminal fibrinogen-like domain, which is the ligand-binding domain (Matsushita et al., 1996). Ficolin-2 trimerizes through the collagen-like domain, and the trimers form higher-order oligomers through disulfide bonds in the N-terminal domain, with the most commonly observed species existing as a 12-mer (a tetramer of trimers) arranged in a bouquet-like structure (Ohashi and Erickson, 2004). Ficolin-2 is produced in the liver and is found primarily in the serum (Matsushita et al., 1996; Ohashi and Erickson, 2004), where it associates with MBL/ficolin-associated serine proteases (MASPs) (Matsushita et al., 2000). Upon ficolin-2 binding to a target, the MASPs become activated and cleave C4 and C2 to form the classical C3 convertase C4b2a and initiate complement activation.

Studies of ficolin-2 function require purified ficolin-2, and several protocols are described to purify ficolin-2 from human serum or plasma using agarose or sepharose beads conjugated to GlcNAc or CysNAc (Krarup et al., 2004; Faro et al., 2008; Matsushita et al., 2014). However, the reported protocols are apparently inefficient, with multiple steps of purification and low reported yields. Recombinant protein expression offers flexibility, and a common approach is the translational fusion of the protein to an affinity tag such as a polyhistidine tag. However, the secreted nature of ficolin-2 requires that the tag be placed at the C-terminal end of the protein. Indeed, commercially available recombinant ficolin-2 from R&D Systems (catalog no. 2428-FC) has a polyhistidine tag at the C-terminus. Since binding of ficolin-2 occurs through the C-terminus, tagging may alter or interfere with ficolin-2 target recognition; indeed, a C-terminally his-tagged ficolin-2 produced in our laboratory failed to bind serotype 11A bacteria (unpublished observation), a natural ficolin-2 target (Brady et al., 2014a). Thus, recombinant ficolin-2 in its natural form is desirable.

However, purification of ficolin-2 can be laborious, and purification of recombinant ficolin-2 presents further challenges due to low concentrations of ficolin-2 in the supernatants, requiring handling and concentration of large volumes of supernatant prior to purification (Lacroix et al., 2009). To avoid these challenges, we have investigated use of bioreactors, which can permit high-concentration yields of secreted proteins. We show that a simple and inexpensive disposable bioreactor produces culture supernatants with high concentrations of ficolin-2 and that ficolin-2 can be directly purified from these supernatants without processing using GlcNAc-agarose, yielding high-purity ficolin-2.

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2. MATERIALS AND METHODS

2.1 Culture conditions

Generation of the human ficolin-2-expressing cell line huf2E by transfection of Chinese Hamster Ovary K1 (CHO) cells with FCN2 cDNA (Genbank accession no. **BC069825**) in pcDNA3.1(−) was described previously (Brady et al., 2014a; Brady et al., 2014b). All cell culture was performed in a humidified 37°C incubator with 5% CO_2. For conventional cell culture, a confluent 150 cm^2 tissue culture flask of huf2E was rinsed with 5 ml Hanks' Balanced Saline Solution without magnesium or calcium (HBSS−/−) and digested with 5 ml trypsin (0.05%)-EDTA solution (Gibco 15400) in HBSS−/− for 5 minutes at 37°C. After digestion, cells were thoroughly resuspended from the flask by pipetting, and 250 µl (1/20 flask, 1.6×10^6 cells) were inoculated into 100 ml fresh Dulbecco's Modified Eagle's Medium/Nutrient Mixture F12 (DMEF, Thermo SH30023) supplemented to 10% heat-inactivated fetal bovine serum (Thermo SH30080) (DMEF-FBS). Geneticin (Life Technologies 10131027) was added to a final concentration of 750 µg/ml. Supernatants were harvested at 14 days, and cells were passaged at harvest as described above.

For bioreactor culture, 1.6×10^6 huf2E cells (prepared as described above) were inoculated into 15 ml DMEF-FBS in the cell chamber of a CELLine Bioreactor Flask (Wheaton Science Products WCL1000-3); 150 ml DMEF was added to the nutrient chamber. Both chambers were supplemented to 750 µg/ml Geneticin. After 14 days of incubation, the nutrient medium was discarded. Cell chamber material (typically 20-25 ml) was removed and centrifuged at 314 × *g* to recover cells. Cells were resuspended in 2 ml DMEF-FBS, and 1/20 cells (~5.2×10^6) were inoculated into 15 ml fresh DMEF-FBS in the cell chamber of the same bioreactor. Fresh nutrient medium and Geneticin were added as described above. It should be noted that the CELLine bioreactor is available with an adherent surface; as CHO cells are compatible with either unit, we used the suspension model and have not tested our protocols with the adherent cell model.

2.2 SDS-PAGE and immunoblotting

Ficolin-2 purity was determined through SDS-10%PAGE of a 15 µl aliquot of the indicated fractions and silver staining using Pierce Color Silver Stain Kit (Thermo Scientific 24597). Ficolin-2-containing fractions were detected through either immunoblot of SDS-10%PAGE of a 5 µl aliquot of the indicated fractions or dot blot of 100 µl serial dilutions of the indicated fractions on a 96-well dot blotting apparatus (Bio-Rad 170-6545). All immunoblots were performed on 0.45 µm nitrocellulose membranes and blocked in 5% powdered skim milk in tris-buffered saline supplemented with 0.05% tween-20. Immunodetection of ficolin-2 was achieved using a biotinylated anti-ficolin-2 antibody (R&D Systems BAF2428) and streptavidinconjugated alkaline phosphatase (Life Technologies 43-4322), both at a dilution of 1:1000, and development using 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium chloride in 1 M tris, pH 8.8.

2.3 Buffers

The dialysis buffer (referred to hereafter as "wash buffer") reported by Lacroix, et al, (145 mM NaCl, 5 mM $CaCl_2$, 20 mM Tris, pH 7.4) was the base for all other buffers (Lacroix et

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al., 2009), but because some solutes were acidic, solutions for purification were made from a 10X wash buffer solution and not adjusted for pH until the final solution was assembled. For elution from GlcNAc column, 500 mM N-acetyl-L-cysteine (CysNAc) was dissolved in 10X wash buffer and water and adjusted to pH 7.4 with 50% w/w NaOH before being brought to 1X ("elution buffer").

2.4 Purification of ficolin-2

All steps were performed at room temperature (~20°C) unless otherwise noted. All column applications were by gravity flow. Ten milliliters of ficolin-2-containing bioreactor supernatants were applied to a 1 ml bed of GlcNAc-agarose (Sigma-Aldrich A2278) (previously equilibrated with 20 ml wash buffer) in a 10 ml polypropylene chromatography column (Bio-Rad 731-1550). The column was washed six times with 10 ml wash buffer prior to five 1-ml elutions with elution buffer. Ficolin-2 containing fractions were determined by dot blotting, pooled, and dialyzed against ≥ 500 volumes wash buffer at 4°C overnight.

Dialyzed ficolin-2 was supplemented with glycerol to 10% and concentrated using 3 kDa molecular weight cutoff microcentrifuge concentrator columns (Millipore UFC500396) to achieve a concentration of ~ 1 mg/ml.

2.5 Quantitation of ficolin-2

Ficolin-2 from supernatants was quantitated absolutely using a human ficolin-2 ELISA kit (Hycult Biotech HK336) or relatively by dot blot. Concentration of recovered ficolin-2 was determined by measuring absorbance at 280 nm in a 1 cm quartz cuvette (using either wash buffer or wash buffer made with 10% glycerol as a blank) assuming an extinction coefficient of 1.767 $(mg/ml)^{-1}$ cm^{-1} calculated using the Protein Calculator (http://protcalc.sourceforge.net/) based on the molecular weight and the method of Gill and von Hippel (Gill and von Hippel, 1989).

2.6 Ficolin-2 functional assays

Ficolin-2 was assayed for binding and complement activation on serotype 11A *Streptococcus pneumoniae* (strain JC03 (Calix et al., 2014)) using flow-cytometric assays as previously described (Brady et al., 2014a; Brady et al., 2014b; Brady et al., 2014c). C1q-depleted serum was purchased from Quidel (A509); normal human serum (NHS) was obtained from a consented, healthy adult donor in glass serum collection tubes under an IRB-approved protocol. NHS was previously determined to have 4.95 μg/ml ficolin-2 (Brady et al., 2014c); commercial C1q-depleted serum is depleted of ficolin-2 (Brady et al., 2014b). Sera were used at 5% final concentration where indicated. Briefly, JC03 was opsonized with ficolin-2 (or in buffer control) at 4 °C for 1 h, washed by centrifugation and resuspension, incubated in the presence of serum (or buffer control) for 1 h at 37°C, and probed for ficolin-2 deposition and complement deposition with specific antibodies before analysis by flow cytometry. C3 and C4 deposition were detected by Pierce Complement C3 Antibody (Thermo Scientific LF-MA0132, used at 1:1000), detected by a phycoerythrin-conjugated goat-anti-mouse secondary antibody (Southern Biotech 1030-09, used at 1:2500), and a fluorescein isothiocyanate-conjugated anti-C4b/C4c antibody (Thermo

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Scientific PA1-28407, used at 1:200). Due to incompatible fluorophores, ficolin-2 binding was detected in parallel in identically prepared samples using the Pierce LFicolin (clone 19) monoclonal antibody (Thermo Scientific ABS005-19-02, used at 1:1000) and a phycoerythrin-conjugated goat-anti-mouse IgG secondary antibody (Southern Biotech 1030-09).

3. RESULTS

3.1 Bioreactor cultivation produces high concentrations of ficolin-2

The low abundance of ficolin-2 in standard CHO cell expression cultures results in the need for extensive processing of supernatants prior to purification (Lacroix et al., 2009). In an effort to further streamline ficolin-2 purification, we grew our ficolin-2-producing cell line, huf2E (Brady et al., 2014a; Brady et al., 2014b), in a CELLine bioreactor using the same passaging/expression schedule as the conventional cultures. Ficolin-2 concentrations from three harvested bioreactor supernatants were compared to three harvested standard culture supernatants by ELISA. Bioreactor supernatants contained 16-39-fold more concentrated ficolin-2 (Figure 1).

3.2 Ficolin-2 can be purified directly from bioreactor supernatants

We hypothesized that in such great abundance, ficolin-2 could be purified directly from bioreactor supernatants without dialysis or concentration. We applied 10 ml (approximately half of a typical supernatant) of bioreactor supernatant to a 1 ml bed of GlcNAc-agarose, washed extensively, and eluted with elution buffer containing 500 mM CysNAc (a flowchart for purification is presented in Figure 2A). As shown in Figure 2, panels B and C, ficolin-2 eluted in high concentrations across the first three elution fractions (1 ml each) before steeply decreasing in the fourth fraction. Much material was retained in the flow-through (Figure 2C, “Post”), suggesting that repeat purifications may provide additional recovery. The eluted ficolin-2 was of high purity, as nearly all species detected in eluates correspond to species detected with a ficolin-2-specific antibody (Figure 2). Interestingly, a band of higher molecular weight than the major species was routinely detected in silver stain and immunoblot; however, it was not consistently observed by coomassie blue staining, suggesting that it is a very minor fraction of the protein (data not shown).

3.3 Purified ficolin-2 is functional

It is essential that purified ficolin-2 is functional. We previously demonstrated that ficolin-2 binds to and can activate complement upon serotype 11A pneumococcus (Brady et al., 2014a). Therefore, we tested our purified, recombinant ficolin-2 for its ability to bind to serotype 11A pneumococci and support complement deposition on their surface in C1q-depleted serum, which is otherwise depleted of ficolin-2 (Brady et al., 2014b). Purified, recombinant ficolin-2 bound to serotype 11A bacteria in a dose-dependent manner (Figure 3) and was able to support C3 and C4 deposition in the presence of C1q-depleted serum (Figure 3A).

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4. DISCUSSION

Existing protocols for the purification of ficolin-2 are inefficient, especially for recombinant ficolin-2, and require many steps of processing and purification. Using CELLine bioreactors, we were able to generate CHO cell supernatants with ficolin-2 concentrations approaching 1 mg/ml – about 20-40 fold more than the conventional cell culture of the same cell line, which provided about 1.5 mg/ml of ficolin-2 per month after purification, with little optimization. Repeated purifications of the starting material are likely to increase recovery, as supernatants still retained ample ficolin-2 after the first purification (Figure 2). The high concentration of ficolin-2 facilitates the purification of high-purity recombinant ficolin-2 without the need for laborious processing. The recombinant ficolin-2 retained its functional capabilities, activating complement on serotype 11A pneumococcus, a natural ficolin-2 target (Brady et al., 2014a). Purified ficolin-2 could also be biotinylated, retaining both its binding and complement-activating abilities (data not shown).

Disposable bioreactors offer a convenient way to increase the relative concentration of recombinant protein to concentrations of other components of the medium that may interfere with purification, especially in an untagged system. For example, the only existing protocol for purification of recombinant ficolin-2 requires concentrating supernatants 20-fold (Lacroix et al., 2009); however, concentrations of FBS corresponding to 4-fold concentrated supernatant (i.e., 40% FBS) are sufficient to partially inhibit ficolin-2 binding (data not shown). Concentrating supernatants also increases the concentration of albumin, which is present in mg/ml quantities and is the most persistent contaminant during ficolin-2 purification. Bioreactor cultivation relieves the need for concentrating supernatants and the complications it introduces.

Bioreactors are a useful solution to the limitations of standard cell culture in the production of recombinant proteins. Large-scale bioreactors are available for industry or industry-oriented laboratories, but two bioreactor systems are suitable for research laboratories. One system is the hollow, fiber-based bioreactor (Fibercell), which circulates medium through a cell chamber in which the cells adhere to a fibrous matrix, and this has been used to produce recombinant proteins (e.g., (Arthos et al., 2002)). However, the system requires other supporting equipment (e.g., pumps), an expense that may deter investigators. Another is the CELLine Bioreactor, which does not require supporting equipment apart from the usual requirements for cell culture. Although its use was reported in 2001 in the production of a recombinant fusion peptide (Docagne et al., 2001), it has not been reported since for use in recombinant protein production and has instead been primarily used for monoclonal antibody production (e.g., (Bruce et al., 2002)). We show that this simple CELLine bioreactor can be useful in producing recombinant proteins for research laboratories with only standard cell culture equipment.

Even under the improved conditions bioreactor cultivation provides, ficolin-2 purification has offered myriad pitfalls. In the development of this protocol, we made additional technical discoveries that may be valuable to others. GlcNAc has been reported to elute ficolin-2 from GlcNAc- or CysNAc-agarose or -sepharose (e.g., refs. (Krarup et al., 2004; Faro et al., 2008; Lacroix et al., 2009; Matsushita et al., 2014)). In our system, GlcNAc in

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concentrations up to 450 mM resulted in diffuse elution, with low concentrations of ficolin-2 across many fractions rather than most eluted material being confined to a few fractions (as in Figure 2); thus, CysNAc is the more efficient eluent in our protocol. Given its efficiency, it may be worthwhile to consider CysNAc in established protocols purifying ficolin-2 from serum or plasma. A previous report of ficolin-2 purification from CHO cell supernatants described concentration of ficolin-2 to 0.1-0.4 mg/ml using spin concentrators (Lacroix et al., 2009). We found that ficolin-2 would precipitate at concentrations presumably above this range in spin concentrators; by supplementing to 10% glycerol prior to concentration, however, we were able to achieve concentrations ~ 1 mg/ml. Even in the presence of 10-20% glycerol, spin concentration reduced the apparent recovery of ficolin-2 (data not shown); we therefore advise that dialysis should be used for buffer exchange and the use of spin concentrators (preferably in the microcentrifuge format, as apparent loss was greater in the 15-ml format) be limited to concentrating the ficolin-2 solution. In addition, we confirmed the reported requirement for 10% FBS in the medium for proper ficolin-2 oligomerization (Lacroix et al., 2009), as reduction to even 5% FBS in the medium resulted in the production of low-order ficolin-2 oligomers as observed using native PAGE (data not shown). Increasing FBS to 20% showed no added benefit.

The study of ficolin-2 has expanded rapidly since ficolin-2 was identified, and purified recombinant ficolin-2 will be a useful tool in further study of this molecule. Bioreactor cultivation eliminates the need for processing large volumes of serum or culture supernatant to purify ficolin-2 through many steps with apparently poor efficiency. Many serum/plasma components, including MBL (Matsushita et al., 2000), bind to GlcNAc-agarose. The use of CysNAc-agarose can provide greater selectivity (Krarup et al., 2004), but the resin is not commercially available, and its generation requires toxic chemicals (Krarup et al., 2004). Use of recombinant protein rather than serum or plasma sidesteps the need for this selectivity.

Pathogenesis research is revealing the involvement of complement pathways in many processes. The complement system has many participating proteins, including several activating molecules and numerous regulators. Recombinant proteins are a powerful tool in dissecting molecular interactions, but efficient protocols for their purification are needed to employ them. Our approach will help to advance studies in this growing field by allowing time, incubator space, and other laboratory resources to be directed towards more fruitful ends and with appropriate modifications may be widely applicable to the *in vitro* production of other related proteins.

ACKNOWLEDGMENTS

This work was supported by NIH grants T32 HL105346 (KAG) and R56 AI031473 (MHN).

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Highlights

- Ficolin-2 is a human serum molecule of increasing immunological interest
- Protocols for purification of recombinant ficolin-2 are laborious and inefficient
- Disposable bioreactor culture allows single-step purification of ficolin-2
- Recovered ficolin-2 is of high-purity and retains complement-activating ability
- Disposable bioreactors should simplify purification of many recombinant proteins

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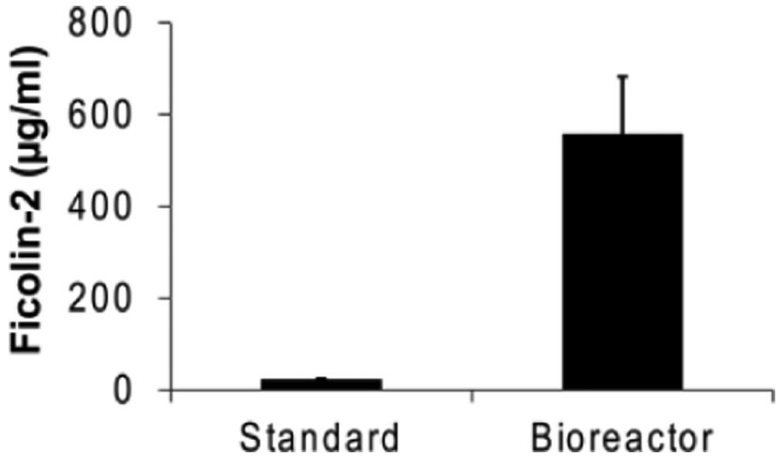

Figure 1.

Recombinant ficolin-2 concentrations in huf2E supernatants. Three independent supernatants from the recombinant ficolin-2-producing cell line huf2E grown under standard culture conditions ("Standard") or in a CELLine bioreactor ("Bioreactor") were assayed for their ficolin-2 content by ELISA as described in *Materials and Methods*. Data are mean + SEM.

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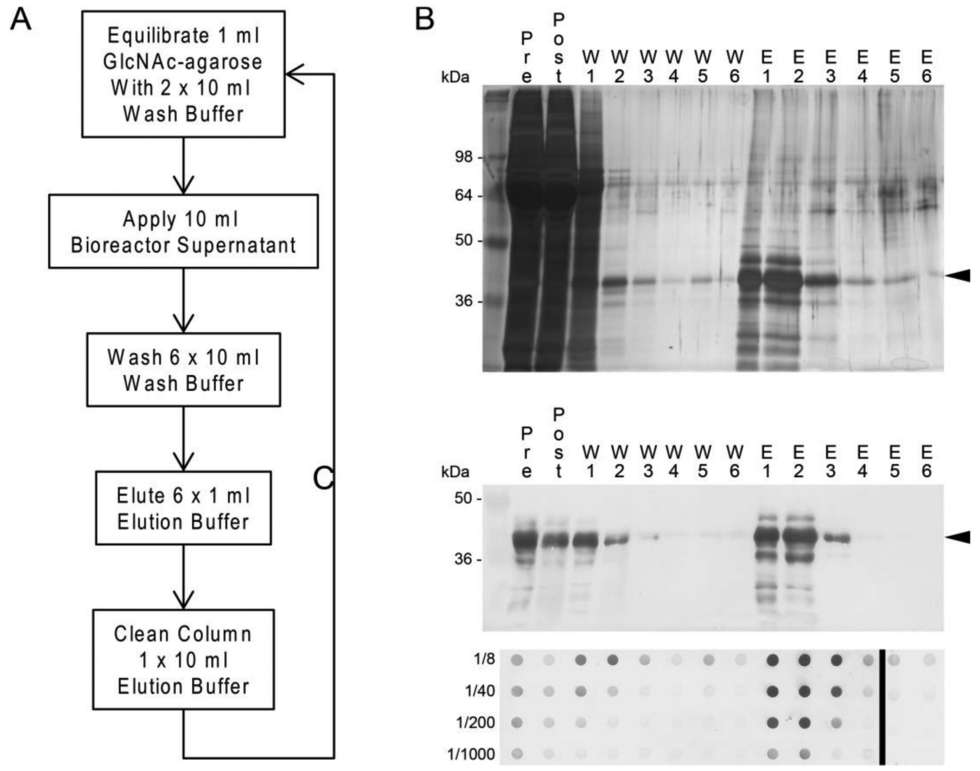

Figure 2.

Ficolin-2 purification from bioreactor supernatants using GlcNAc-agarose. A, schematic for ficolin-2 purification. B, silver stain of 15 μl aliquots of fractions from purification. Arrows indicate the major ficolin-2 bamd C, upper, immunoblot of 5 μl aliquots of purification samples; lower, dot immunoblot of 100 μl of purification samples at the indicated dilution. The vertical black line marks where another portion of the same dot blot is displayed beside the other portion. Fractions: Pre, starting material; Post, flow-through from GlcNAc column; W1-6, washes 1-6; E1-6, elutions 1-6.

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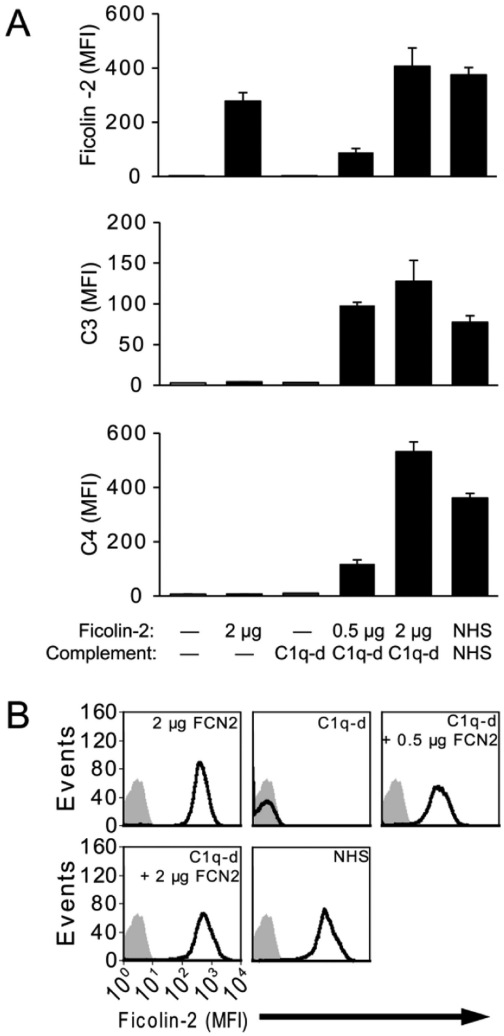

Figure 3.

Purified recombinant ficolin-2 is functional. A, detection of ficolin-2, C3, and C4 on serotype 11A bacteria incubated with or without purified recombinant ficolin-2 and/or 5% serum as a complement source, as indicated, for 1 h . All events with forward scatter and side scatter ≥ 10 were analyzed. Data shown are geometric mean fluorescent intensities + SD of three independently-prepared replicates. Due to incompatible fluorophores with C3 detection, ficolin-2 binding was analyzed in identically-prepared wells on the same plate at the same time. B, representative histograms of ficolin-2 binding from panel A. Gray shading indicates no ficolin-2, no serum control. C1q-d, C1q-depleted serum; NHS, normal human serum; FCN2, ficolin-2.