Journal of Immunological Methods 362 (2010) 204–208

Contents lists available at ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Technical Note

A simple two-step purification procedure for the iC3b binding collectin conglutinin

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ARTICLE INFO

Article history:
Received 4 August 2010
Accepted 1 September 2010
Available online 9 September 2010

Keywords:
C-type lectin
Collectin
Conglutinin
Purification
Complement activation

ABSTRACT

Bovine conglutinin is a serum protein involved in innate immunity. It binds calcium dependently to iC3b, a product of the complement component C3 deposited on cell surfaces, immune complexes or artificial surfaces after complement activation. We here present a simple and efficient two-step procedure for the purification of conglutinin. In the first step, bovine serum is incubated with non-coupled chromatographic TSK beads at 37 °C to allow complement activation and iC3b deposition on the beads and subsequent binding of conglutinin to iC3b. Conglutinin is then eluted from the beads by EDTA. In the second step, conglutinin is separated from iC3b and IgM by ion-exchange chromatography. This purification procedure yielded 81 μg of conglutinin per ml of serum with a recovery of 61.2%. Surface plasmon resonance analysis showed that the purified conglutinin had a high affinity for mannan ($K_d = 2.3 - 3.2$ nM). SDS-PAGE and time-resolved immunofluorometric assays showed that the conglutinin was not contaminated with other serum collectins such as collectin-43 or mannan-binding lectin.

1. Introduction

Bovine conglutinin (BK) is a member of the collectin family of proteins composed of C-type lectin domains connected to collagen-like regions. The abundant form of BK is a dodecamer of 43 kDa monomers arranged in a flexible cross-like structure of four homotrimers. Conglutinin binds microorganisms either directly or via the complement degradation product iC3b through the lectin domains. The binding leads to agglutination of the microorganisms and it promotes phagocytosis, and BK thereby plays an important role in the bovine innate immune defence system (for review see Holmskov (2000)). We have previously shown that serum concentrations of BK vary among individual cows, that the levels are genetically determined and that low BK serum concentrations predispose to infection (Holmskov et al., 1998). These results lead to a renewed interest in conglutinin, especially in cattle breeding programs. We have developed a time-resolved immunofluorometric assay for large-scale measurement of serum BK (Krogh-Meibom et al., 2004) and purified BK was needed to establish the assay standard. Also, purified BK is used for the conglutination test to study iC3b coated immune complexes (Tsai et al., 1998) and for purification of immune complexes (Casali and Lambert, 1979). We here describe a simple two-step purification procedure for BK based on iC3b affinity chromatography and ion-exchange chromatography.

Abbreviations: BK, bovine conglutinin; CL-43, collectin-43; EDTA, ethylenediamine tetra-acetic acid; HBS, HEPES buffered saline; MBL, mannan-binding lectin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SPR, surface plasmon resonance; TBS, tris-buffered saline; TRIFMA, time-resolved immunofluorometric assay.

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doi:10.1016/j.jim.2010.09.010

2. Materials and methods

2.1. Buffers

Tris buffered saline (TBS): 10 mM Tris-Base (Tris[hydroxymethyl]aminomethane, Sigma, St. Louis, MO, cat. no. T-1503), 140 mM NaCl, 0.05% emulfogen (polyoxyethylene 10 tridecyl ether, Sigma, cat. no. P-2393), pH 7.4; TBS–Ca: TBS with 5 mM $CaCl_2$; TBS–Ca 1 M NaCl: TBS–Ca containing a total of 1 M NaCl; TBS–EDTA: TBS with 5 mM EDTA; SDS-PAGE sample buffer: 10 mM Tris–HCl, 1 mM EDTA, 10% SDS (Sigma, cat. no. L-4509), 2% Bromophenol Blue; SDS-PAGE running buffer: 14% glycin, 3% Tris-Base, 1% SDS; piperazine loading buffer: 20 mM piperazine (diethylenediamine, Sigma, cat. no. P4, 590-7), 50 mM NaCl, 5 mM EDTA, 0.05% emulfogen, pH 6.2; HEPES buffered saline (HBS): 10 mM HEPES (N-[2-hydroxymethyl]piperazine-N′-[2-ethanesulfonic acid], Sigma, cat. no. H-7523), 150 mM NaCl, 0.005% surfactant P-20 (BIAcore, Herts, U.K., cat. no. BR-1000-54), 0.02% NaN_3, pH 7.4; HBS–Ca: HBS with 5 mM $CaCl_2$ and HBS–EDTA: HBS with 5 mM EDTA.

2.2. Serum isolation and storage

Blood from two cows was randomly collected at the local abattoir and allowed to clot overnight at 4 °C. Serum was isolated by centrifugation at 1000×*g* for 30 min. Sera from the two cows were pooled and stored in 500 ml aliquots at −20 °C until use. The BK concentration in the pooled serum was 132 μg/ml. The concentrations in adult cows vary from approximately 100 ng/ml to above 1 mg/ml with a geometrical mean of approximately 40 μg/ml (Krogh-Meibom et al., 2004).

2.3. Purification of conglutinin

The chromatographic beads (TSK, Toyopearl HW-75F, Sigma, cat. no. 8-07469) were washed extensively with water followed by TBS–Ca buffer on a glass filter. Serum was thawed overnight at room temperature, centrifuged for 30 min at 10,000×*g*, and filtered through a glass filter. A volume of 100 ml serum was incubated with 60 ml of TSK beads for 2.5 h at 37 °C in a 200 ml glass flask. The TSK beads were gently resuspended every 30 min. The TSK beads were packed under flow on a computer-monitored FPLC system (FPLCdirector Version 1.3, Amersham Biosciences, Uppsala, Sweden) and washed first with TBS–Ca 1 M NaCl until the absorbance at 280 nm was below 0.1, and then equilibrated with TBS–Ca buffer until the absorbance reached the baseline. The column was then eluted with TBS–EDTA, and the fractions containing protein were pooled. The EDTA eluate was diluted 1/4 in piperazine loading buffer pH 6.2 and applied to a 1 ml Mono Q anion-exchange column (Amersham Biosciences, cat. no. 17-0546-01) at a flow rate of 0.5 ml/min. After washing with loading buffer, retained protein was eluted with a linear gradient from 50 to 375 mM NaCl over 30 column volumes in piperazine loading buffer pH 6.2. Samples of the starting materials and from each purification step were collected and analysed by SDS-PAGE and in the BK–TRIFMA-assay as described below.

As a control, a volume of 100 ml serum was heat-inactivated for 1 h at 56 °C and used in the purification procedure described above. As further controls, 1 ml TSK was incubated with purified BK (100 μg/ml) for 2.5 h at 37 °C, and 1 ml volumes of TSK that had previously been incubated with serum and eluted with TBS–EDTA were incubated with either purified BK or heat-inactivated serum. The TSK beads were then washed with TBS–Ca 1 M NaCl and with TBS–Ca by repeated suspension, centrifugation and removal of the supernatant. Finally, the beads were resuspended in 1 ml TBS–EDTA (10 mM EDTA) and the supernatants were analysed by SDS-PAGE and by the BK–TRIFMA-assay.

2.4. SDS-PAGE

Electrophoresis was performed on 4–20% polyacrylamide gradient gel and samples were reduced by heating at 100 °C for 2 min in 50 mM dithiothreitol diluted in SDS-PAGE sample buffer and alkylated by the addition of iodoacetamide to a concentration of 100 mM. Non-reduced samples were heated in SDS-PAGE sample buffer with 2 mM iodoacetamide. Samples of 10 μl were loaded per lane. Protein bands were detected with Coomassie Brilliant Blue. The molecular mass markers were those of the Mark 12 standard (Invitrogen NOVEX, San Diego, CA, cat. no. LC5677) or Precision Protein Standards (Bio-Rad, cat. no. 161-0362).

2.5. N-terminal amino acid sequencing

Samples were reduced and run on a 10% NOVEX Bis-Tris NuPAGE precast gel (Invitrogen NOVEX) at 200 mA per gel in a NOVEX XCell II Mini-cell gel apparatus. The gel was electroblotted to a NOVEX 0.2 μm PVDF membrane in a NOVEX blot module. The membrane was then stained with Coomassie Brilliant Blue. The bands of interest were excised from the PVDF membrane and washed extensively with 10% methanol in water prior to sequencing. They were then sequenced on an Applied Biosystems 494A 'Procise' protein sequencer (Applied Biosystems, Warrington, U.K.) using standard sequencing cycles.

2.6. Amino acid analysis

Samples were run on an ABI 420A analyser (PE Biosystems, Warrington, U.K.) following hydrolysis for 24 h in 5.7 M hydrochloric acid at 110 °C. Data were integrated using Dionex Chromeleon v6.4 software (Dionex, Macclesfield, U.K.).

2.7. Time-resolved immunofluorometric assay (TRIFMA) for conglutinin

A BK–TRIFMA-assay was used to estimate the recovery of BK (Krogh-Meibom et al., 2004). Briefly, wells of microtiter plates (MaxiSorp, Nunc, Kamstrup, Denmark) were coated with anti-BK polyclonal antibodies by overnight incubation. The wells were washed and incubated overnight with dilutions of BK containing samples. The wells were then incubated stepwise with an anti-BK monoclonal antibody and a biotin-labelled subclass specific rabbit anti-mouse antibody, followed by Eu^{3+} chelates coupled to streptavidin. After