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Research paper

Calcium-sensitive immunoaffinity chromatography: Gentle and highly specific retrieval of a scarce plasma antigen, collectin-LK (CL-LK)☆

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ABSTRACT

Immunoaffinity chromatography is a powerful fractionation technique that has become indispensable for protein purification and characterization. However, it is difficult to retrieve bound proteins without using harsh or denaturing elution conditions, and the purification of scarce antigens to homogeneity may be impossible due to contamination with abundant antigens. In this study, we purified the scarce, complement-associated plasma protein complex, collectin LK (CL-LK, complex of collectin liver 1 and kidney 1), by immunoaffinity chromatography using a calcium-sensitive anti-collectin-kidney-1 mAb. This antibody was characterized by binding to CL-LK at hypo- and physiological calcium concentrations and dissociated from CK-LK at hyperphysiological concentrations of calcium. We purified CL-LK from plasma to a purity of 41% and a yield of 38%, resulting in a purification factor of more than 88,000 in a single step. To evaluate the efficiency of this new purification scheme, we purified CL-LK using the same calcium-sensitive mAb in combination with acidic elution buffer and by using calcium-dependent anti-CL-K1 mAbs in combination with EDTA elution buffer. We found that calcium-sensitive immunoaffinity chromatography was superior to the traditional immunoaffinity chromatographies and resulted in a nine-fold improvement of the purification factor. The technique is applicable for the purification of proteins in complex mixtures by single-step fractionation without the denaturation of eluted antigens, and it allows for the purification of scarce proteins that would have otherwise been impossible to purify and, hence, to characterize. This technique may also potentially be applied for the purification of proteins that only interact with calcium ions at hyperphysiological concentrations.

1. Introduction

Immunoaffinity chromatography is a powerful fractionation technique wherein the exquisite specificity and high affinity of an antibody–antigen interaction allow for a highly selective adsorption of a given protein of interest. By simply applying the protein mixture to an immobilized antibody, removing unbound material by washing and eluting the bound protein (antigen) by appropriate elution, it is possible to achieve a purification factor >1000 for most common antigens. Many proteins can be purified easily and efficiently by traditional immunoaffinity chromatography, and the technique has

Abbreviations: CL-K1, collectin kidney 1; CL-L1, collectin liver 1; CL-LK, collectin LK (heteromeric complex of CL-L1 and CL-K1); CRD, carbohydrate recognition domain; MBL, mannan-binding lectin; MASP(s), MBL-associated serine protease(s), OPD, ortho-phenylenediamine.

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proven its worth in laboratory settings and even in large-scale purifications of therapeutic proteins (Subramanian, 2002; Moser and Hage, 2010; Sheng and Kong, 2012).

However, when untagged, native proteins are purified from a complex mixture, such as plasma or serum, serial steps of purification are usually required to obtain sufficient purity. This is because many abundant plasma proteins bind non-specifically to the column matrix or to the conjugated antibodies; often in a calcium dependent manner. Because most commonly applied elution buffers non-specifically elute the proteins by exploiting low pH or high ionic strength, the eluted product may be of low purity, which severely complicates the procedures and decreases the success rate of purification of scarce antigens from complex mixtures. In theory, a much higher purity can be obtained using specific elution conditions that specifically dissociate the antigen from the antibody without disturbing the non-specific binding of contaminants to the column.

Collectin K1 (CL-K1) is a scarce plasma protein that can be found in the circulation in the form of heteromeric complexes (CL-LK) with another collectin, collectin L1 (CL-L1) (Ohtani et al., 1999; Keshi et al., 2006; Henriksen et al., 2013a). Collectins are a group of innate immune proteins that are structurally defined by inclusion of a collagen-like region and a C-type lectin domain, which is also referred to as carbohydrate recognition domain (CRD) (Selman and Hansen, 2012). Well-characterized collectins include mannan-binding lectin (MBL), surfactant protein A (SP-A) and surfactant protein D (SP-D) (Holmskov et al., 2003). CL-LK has been shown to interact with MBL-associated serine proteases (MASPs) and can mediate complement activation upon binding to suitable microbial and endogenous ligands (Hansen et al., 2010; Henriksen et al., 2013b). CL-LK is found as an oligomer with up to six subunits, in which each subunit is made of three CL-L1/CL-K1 polypeptide chains that are linked together by di-sulfide bridges (Henriksen et al., 2013a). Novel studies show that human CL-K1 and MASP-3 deficiency or malfunction is strongly associated with the developmental midline-related defect syndrome, designated the 3MC syndrome, and these findings suggest an additional role for CL-LK in developmental processes (Rooryck et al., 2011). Because the plasma concentration of CL-LK, as measured by a CL-K1-specific ELISA, is approximately only 0.3 μg/mL (Selman et al., 2012; Yoshizaki et al., 2012), traditional purification schemes fail to provide pure preparations of CL-LK in a necessary yield for functional and structural studies.

In this study, we purified CL-LK from plasma using a calcium-sensitive mAb in combination with calcium buffer to specifically elute CL-LK. This new method prevents the elution of contaminants that are non-specifically bound to the column and further allows for the removal of calcium-dependently bound contaminants by enabling loading and washing in buffers with EDTA prior to elution. We found that purification of CL-LK from plasma using calcium-sensitive immunoaffinity chromatography is an overall superior method compared to traditional immunoaffinity chromatography.

2. Materials and methods

2.1. Reagents and buffers

Unless otherwise stated, reagents were obtained from Sigma-Aldrich (Broendby, Denmark). The following reagents were used: TBS (20 mM Tris and 125 mM NaCl, adjusted to pH 7.4 with HCl), EDTA wash buffer (TBS with 5 mM EDTA, 50 mM NaCl, 0.05% Emulfogen (polyoxyethylene 10 tridecyl ether)), MASP dissociation buffer (20 mM Tris, 10 mM EDTA, 1 M NaCl, pH 7.4), TBS wash buffer (TBS with 50 mM NaCl), calcium elution buffer (TBS with 100 mM $CaCl_2$), acidic elution buffer (50 mM glycine, pH 2.3), calcium wash buffer (TBS with 5 mM $CaCl_2$, 50 mM NaCl, 0.05% Emulfogen), EDTA elution buffer (TBS, 10 mM EDTA), coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6), TBS/EDTA/Em (TBS with 5 mM EDTA, 0.05% Emulfogen) and TBS/Ca/Em (TBS with 2 mM $CaCl_2$, 0.05% Emulfogen).

2.2. Generation of monoclonal anti-CL-K1 antibodies

Monoclonal mouse anti-human CL-K1 Abs were raised against full-length recombinant human CL-K1 expressed in DG44 CHO cells and purified from serum-free hybridoma media by Protein G affinity chromatography essentially as described previously (Hansen et al., 2010; Selman et al., 2012). Mice were immunized with 20 μg recombinant CL-K1 dissolved in TBS with either 5 mM EDTA or 10 mM $CaCl_2$. Prior to immunization recombinant CL-K1 was mixed with 1% $Al(OH)_3$ and emulsified in equal volumes of a mixture of Freund's complete/incomplete adjuvant (ratio 1:10). To avoid the formation of large granulomas only a small amount of Freund's complete adjuvant (10%) was used for the primary immunization. After three weeks, booster injections were given by the use of $Al(OH)_3$ and Freund's incomplete adjuvant and repeated once after two weeks, before intravenous immunization and fusion of spleen cells.

2.3. Characterization of monoclonal anti-CL-K1 antibodies

ELISA was used to determine the reactivity of the anti-CL-K1 mAbs in the absence or presence of calcium. Maxisorp plates (96-well, Nunc, Roskilde, Denmark) coated with purified recombinant CL-K1 (2 μg/mL, overnight at 4 °C) were incubated with 0.5 μg/mL anti-CL-K1 mAbs (2 h at room temperature) diluted in TBS with 0.05% emulfogen and various concentrations of EDTA, $CaCl_2$, or $MgCl_2$ and different concentrations of NaCl to compensate for differences in ionic strength. Following washing, all plates were incubated with HRP-conjugated rabbit anti-mouse IgG antibody (Millipore, CA, diluted 1:2000) and developed using ortho-phenylenediamine (OPD) with H_2O_2 as substrate.

2.4. Coupling of anti-CL-K1 mAbs to Sepharose

Monoclonal antibodies were conjugated to CNBr-activated Sepharose 4B according to the manufacturer's recommendations (GE Healthcare, Broendby, Denmark; 5 mg mAb/mL of beads).

2.5. Preparation of plasma and serum

Outdated plasma stabilized with citrate phosphate dextrose was routinely obtained from Odense University Hospital and frozen at −80 °C until use. For the comparison of purification methods, plasma was thawed at 4 °C overnight, pooled and divided into four batches of 1.5-L plasma. Each batch was