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

Maiken L. Henriksen*, Kirstine L. Madsen, Karsten Skjoedt, Soren Hansen

Department of Cancer and Inflammation Research, Institute of Molecular Medicine, University of Southern Denmark, J. B. Winsloews Vej 21.1, DK-5000 Odense, Denmark

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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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* Corresponding author. Tel.: +45 65503778.

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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-sulphide 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

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refrozen until further use. For each particular purification, plasma was thawed immediately before use, centrifuged at 17,000 ×*g* for 30 min and filtered through a glass-fibre filter (Satorius Stedim Biotech, Goettingen, Germany) before the addition of 10 mM EDTA, 0.05% Emulfogen and 0.05% sodium azide. EDTA was added to the plasma to favour the specific, calcium-free conformation of CL-K1. For purifications in the presence of calcium, serum was applied. Serum was pooled into two batches of 1100 mL and treated like plasma but without the addition of EDTA.

2.6. Purification using the calcium-sensitive mAb

Undiluted plasma was applied to a column (8 mL) that was coupled with a calcium-sensitive anti-CL-K1 mAb (Hyb 15–8). The column was washed sequentially with EDTA wash buffer and MASP dissociation buffer. The latter step included a prolonged incubation in the form of a "stop" for 1 h after applying the buffer. Subsequently, the buffer was changed to TBS wash buffer before eluting CL-LK with calcium buffer. A 4-hour "stop" was included directly after application of the calcium buffer and before elution of CL-LK. To compare this method to traditional purification procedures, CL-LK was purified from plasma using the same calcium-sensitive antibody and procedures as above using an acidic elution buffer instead of calcium buffer. For comparison, a total of four purifications were performed using the calcium-sensitive mAb; two wherein bound proteins were eluted with calcium elution buffer, and two were eluted with acidic elution buffer.

2.7. Purification using calcium-dependent mAbs

Serum was applied to a column (8 mL) that was coupled with a mixture of calcium-dependent anti-CL-K1 mAbs (Hyb 14–1, 14–6, 14–10 and 14–20). The column was washed with calcium wash buffer and TBS wash buffer before eluting CL-LK with EDTA elution buffer. A 4-hour "stop" was included directly after application of the EDTA buffer before the final elution of CL-LK. Two purifications were performed using the calcium-dependent mAb.

2.8. SDS-PAGE and silver- and Coomassie staining

SDS-PAGE was performed using pre-cast NuPAGE® Novex gels and running buffers from Invitrogen (Naerum, Denmark) according to the manufacturer's recommendations. Protein bands were visualized by silver staining according to the method of Nesterenko et al. with previously described modifications (Nesterenko et al., 1994; Selman et al., 2008). Coomassie staining was performed using the Simply Blue Safe stain according to the manufacturer's recommendations (Life Technologies, Naerum, Denmark).

2.9. ELISA for the quantification of collectin K1

Two different monoclonal anti-CL-K1 antibodies were used in ELISAs for coating and detection to quantify the amount of CL-K1 in plasma and in purified samples, as previously described (Selman et al., 2012). Briefly, microtiter plates coated with anti-CL-K1 mAb (5 μg/mL, Hyb 11–2) were incubated with dilutions of CL-LK overnight at 4 °C, and bound complexes were detected using biotin-coupled anti-CL-K1 mAb (0.5 μg/mL, Hyb 14–29). The signals were developed using HRP conjugated streptavidin and OPD.

2.10. Quantification of total protein

The concentration of total protein in plasma/serum and purified samples was determined using the BCA Protein Assay (Pierce™, Thermo Scientific), Coomassie (Bradford) Protein Assay (Pierce™, Thermo Scientific) and by spectrophotometry using an average extinction coefficient of 1.0 $(g/L)^{-1}\,cm^{-1}$. The results are given as the mean values of all three methods.

3. Results

3.1. Characterization of anti-CL-K1 mAbs

Monoclonal antibodies raised against recombinant CL-K1 were initially screened in parallel in buffers containing either EDTA or calcium. The specificity of selected mAbs with relatively high affinity for CL-K1 was further examined using ELISA, wherein the mAbs were incubated in CL-K1-coated microtiter wells in buffers of different composition with regard to calcium (Fig. 1). To clarify if changes in affinity were caused by increased calcium concentrations or by an increase in ionic strength, some buffers were also adjusted with NaCl to obtain identical ionic strengths. Anti-CL-K1 mAb Hyb 15–8 bound equally well to CL-K1 in the absence of $CaCl_2$ or at physiological concentrations of $CaCl_2$. However, at concentrations of 100 mM $CaCl_2$, the binding was strongly decreased. Compared to other buffers with identical ionic strength, this decrease in affinity was due to a combination of an increase in the $CaCl_2$ concentration and in the ionic strength. In contrast to Hyb 15–8, the reactivity of the following mAbs: Hyb 14–1, 14–6, 14–10 and 14–20 completely depended on the presence of calcium.

3.2. Purity and yield of CL-LK

CL-LK was purified from plasma by immunoaffinity chromatography using the calcium-sensitive mAb Hyb 15–8 combined with calcium buffer to specifically elute CL-LK from the column (Fig. 2). A concentration of 61.4 μg/mL purified CL-LK was achieved (Table 1) and a total amount of 123 μg CL-LK was obtained, which corresponded to 38.2% of the initial content of CL-LK in the plasma (Table 2). The total amount of protein in the purified sample was estimated to be 298 μg, resulting in a CL-LK purity of 41.3%. CL-LK was also purified from plasma using acidic buffer for elution, instead of the specific calcium elution buffer, and a concentration of 22.4 μg CL-LK/mL was obtained, with a total amount of CL-LK of 224 μg and a total protein content of 4980 μg (Table 1), giving a purity and yield of 4.5% and 73.4%, respectively (Table 2). Thus, a final purification factor of 88,400 resulted when using calcium buffer for specific elution. Using acidic buffer, we obtained an approximately nine-fold lower purification factor of 9900. The efficiency of purifying CL-LK using the calcium-sensitive mAb Hyb 15–8 was also compared to purifying CL-LK using a mix of calcium-dependent mAbs (a mix of hyb 14–1, 14–6, 14–10 and 14–20) in combination with specific EDTA elution and a concentration of only 6.1 μg CL-LK/mL was obtained by this

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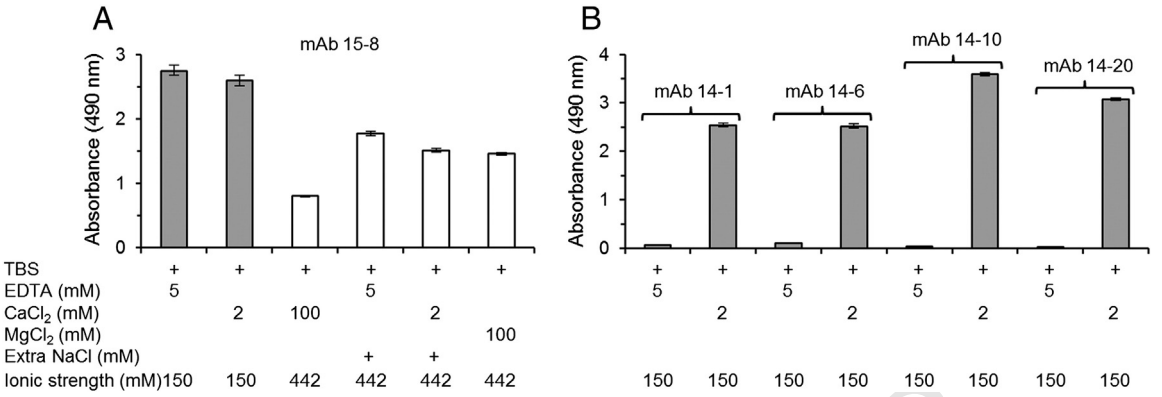

Fig. 1. Binding characteristics of mAbs. (A) Detailed binding characteristics of the calcium-sensitive anti-CL-K1 mAb, Hyb 15–8. (B) Binding characteristic of the calcium-dependent anti-CL-K1 mAbs, Hyb 14–1, 14–6, 14–10 and 14–20. mAbs were incubated in microtiter wells coated with recombinant CL-K1 in the given buffer composition. Binding was detected using HRP-conjugated rabbit anti-mouse IgG pAb. The results shown in open columns were analysed using buffers adjusted to an identical ionic strength corresponding to 442 mM NaCl. The experiment was performed two times, and error bars refer to the maximum and minimum of triplicate measurements.

method (Table 1). The relatively low amount of CL-LK by this EDTA elution resulted in a yield of only 6.0% and a purity of only 1.5% (Table 2).

3.3. SDS-PAGE of purified CL-LK

The purity of CL-LK obtained using the three different purification methods was visualized by SDS-PAGE and silver or Coomassie staining (Fig. 3). The samples were diluted so that approximately 50 ng of CL-LK from each purification method was analysed. Both silver and Coomassie staining showed that the purity of CL-LK was several-times higher when using the calcium-sensitive mAb (Hyb 15–8) in combination with specific calcium elution compared to using acidic elution or calcium-dependent mAbs with EDTA buffer for elution; the latter had the lowest purity of CL-LK. The three major contaminants in the final preparation obtained by calcium elution of Hyb 15–8 were determined using mass spectrometry-assisted mass fingerprinting and found to be heavy chain IgG (~53 kDa) and albumin (~68 kDa) (Henriksen et al., 2013a). By eluting through a micro column composed of a mixture of protein A/G/L and anti-albumin Sepharose, these contaminants could be removed (Fig. 3).

4. Discussion

During the generation of monoclonal antibodies against CL-K1, we characterized an antibody with sensitivity to high concentrations of calcium ions, which led to the dissociation of the antibody–antigen complex. We exploited this characteristic

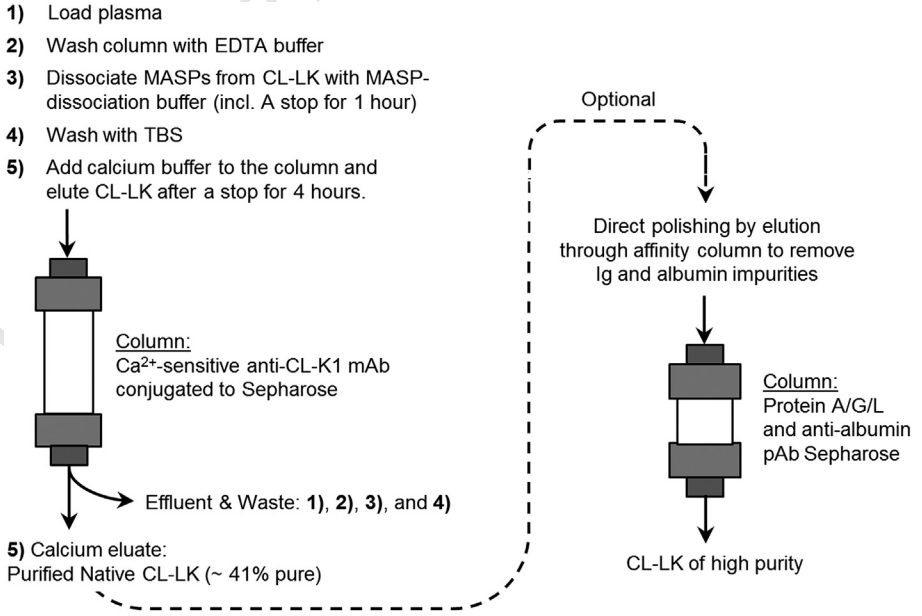

Fig. 2. Flow chart for the purification of CL-LK from plasma by calcium-sensitive immunoaffinity chromatography. MASP-dissociation buffer refers to TBS with 10 mM EDTA and 1 M NaCl. The precise compositions of other buffers and details for the chromatography are provided in the Materials and methods.

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Table 1
Purification of CL-LK from plasma or serum.

Purification method	Source and CL-LK quantity	Volume (mL)	CL-K1 conc. (μg/mL)	Total protein conc. (μg/mL)	Amount of CL-K1 (μg)	Amount of total protein (μg)
Ca^{2+}-sens. mAb; Ca^{2+} elution	Plasma	1400	0.230 ± 0.001	$49.2 \times 10^3 \pm 1.8 \times 10^3$	322	68.9×10^6
	purified CL-LK	2	61.4 ± 6.2	149 ± 1	123	298
Ca^{2+}-sens. mAb; acidic elution	Plasma	1400	0.218 ± 0.002	$47.9 \times 10^3 \pm 1.4 \times 10^3$	305	67.1×10^6
	Purified CL-LK	10	22.4 ± 1.2	498 ± 0	224	4980
Ca^{2+}-dep. mAbs; EDTA elution	Serum	1075	0.281 ± 0.005	$63.0 \times 10^3 \pm 1.3 \times 10^3$	302	67.7×10^6
	Purified CL-LK	3	6.1 ± 0.2	394 ± 4	18	1182

Ca^{2+}-sens. mAb, CALCIUM-sensitive mAb 15–8; Ca^{2+}-dep. mAbs, calcium-dependent mAbs, 14–1, −6, −10 and −20. CL-LK concentrations were measured using ELISA, and the total protein concentration was estimated as the mean of three different methods (BCA, Bradford and optical density). The ± variations specify the maximum and minimum concentrations from two individual purifications performed using each of the three methods.

and succeeded in purifying native CL-K1 of high purity from plasma using calcium-sensitive immunoaffinity chromatography, albeit only a low initial plasma concentration of only 300 ng/mL. This led to our previously reported findings that CL-K1 was found in the circulation in the form of disulphide-stabilized, heteromeric complexes (CL-LK) with another collectin, CL-L1 (Henriksen et al., 2013a). By refining the procedure and comparing yields and purity, we found that calcium-sensitive immunoaffinity chromatography may be as much as nine-fold more efficient in relation to the purification factor than traditional immunoaffinity chromatography and could serve as a new tool for purifying scare antigens from complex mixtures.

In terms of purity, we refined the procedure for calcium-sensitive immunoaffinity chromatography to provide a purity of 41% using a single fractionation step. Taking into consideration that CL-LK is a scarce antigen and that we only applied a single step to achieve this purity, this is a relatively high purity. Further purity may be achieved using elution through a column conjugated to a combination of Protein A, G and L and anti-albumin antibodies, but this step was omitted to compare methods. However, this step elegantly illustrates that the method is versatile and that the eluate can readily proceed to further fractionation steps, if required, without the need for neutralization, renaturation or dialysis. However, gentle calcium elution was less efficient, in relation to the yield, than the acidic elution and resulted in a final yield of only 38.2% compared to 73.4%, respectively. In most situations, a nine-time higher purity by far outweighs a lower yield. It should be mentioned that the yield of the calcium elution was biased by the fact that adjacent fractions with low concentrations of CL-LK were not included in the calculations (data not shown).

Table 2
Comparison of the yield, purity and purification factor.

Purification method	Yield (%)	Purity (%)	Purification factor
Ca^{2+}-sens. mAb Ca^{2+} elution	38.2	41.3	88,400
Ca^{2+}-sens. mAb; acidic elution	73.4	4.5	9900
Ca^{2+}-dep. mAbs; EDTA elution	6.0	1.5	3400

Ca^{2+}-sens. mAb, calcium-sensitive mAb 15–8; Ca^{2+}-dep. mAbs, calcium-dependent mAbs, 14–1, −6, −10 and −20. The yield is determined as the percentage of purified CL-LK compared to the initial amount present in the plasma pool. The purity is determined as the percentage of CL-LK in relation to the total amount of protein in the purified sample. The purification factor is determined as the ratio between the purity of CL-LK (μg CL-LK/mL per μg total protein/mL) in the plasma or serum and after purification.

Furthermore, due to the high oligomeric nature of CL-LK and potentially high avidity interaction with the conjugated antibodies, a small fraction of the largest oligomers of CL-LK was retained on the column during calcium elution. This fraction of CL-LK could subsequently be eluted by acidic elution (not shown). We expect this problem to be irrelevant for the purification of other monomeric or low-oligomeric proteins. The application of a mixture of five different calcium-dependent mAbs, combined with EDTA elution, performed very poorly. The total amount of proteins in the eluted fractions showed that a large amount of impurities from plasma bound to the column in a calcium-dependent manner and thus contributed as a great source of contamination in the eluate. The presence of so many bound impurities onto the column is likely to decrease the capacity of the column. These impurities were most likely removed by loading in the presence of EDTA and by inclusion of an EDTA-washing step in traditional and calcium-sensitive immunoaffinity chromatographies and could explain why

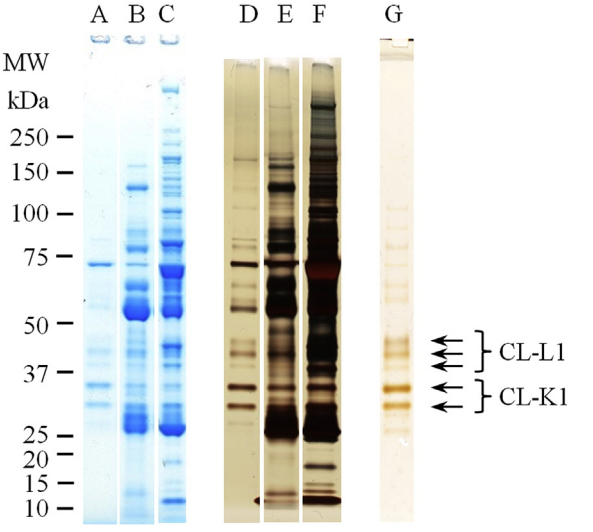

Fig. 3. SDS-PAGE of purified CL-LK. Immunoaffinity purification by calcium-sensitive mAb (Hyb 15–8) and elution with calcium buffer (A, D) or acidic elution (B, E). Immunoaffinity purification by calcium-dependent mAbs and elution with EDTA buffer (C, F). Fractions in A–C were stained by Coomassie brilliant blue, and those in D–G were submitted to silver staining. Approximately 50 ng of CL-LK was loaded in each well. As a polishing step, calcium elution could be performed through a micro column of 0.5 mL Sepharose coupled with protein A/G/L and anti-albumin mAb (G).

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calcium-dependent immunoaffinity chromatography was inferior in terms of both yield and purity. The less successful application of calcium-dependent mAbs may also be due to a low selectivity and affinity of the calcium-dependent mAbs (not analysed), although we used a mixture of mAbs to ensure an overall high avidity of the column.

Gentle elution strategies for immunoaffinity chromatography have previously been exploited with success in the purification of labile proteins and multisubunit complexes in which high yield, purity and conserved enzyme activity were achieved (Moser and Hage, 2010). A well-described method was based on the selection and application of polyol-responsive mAbs that bound strongly to their antigens under physiological conditions but dissociated in the presence of TBS with 30% propylene-glycol and 0.7 M NaCl (Rhemrev-Boom et al., 2001).

Using calcium-sensitive antibodies also allows for the use of plasma without extensive preparation, in contrast to using calcium-dependent antibodies, in which it is necessary to add calcium to the plasma and perform extensive coagulation procedures. In many situations, the usage of plasma, compared to serum, is advantageous. For example, when purifying zymogens related to, e.g., the coagulation and fibrinolytic system, the non-activated forms of the involved enzyme are better conserved in plasma than in serum.

Our initial characterization of the calcium-sensitive mAb showed that binding to CL-K1 was affected by hyperphysiological concentrations of calcium due to a combined effect of increased calcium concentration and increased ionic strength. It is actually not clear whether the calcium ions interfere with the antigen or antibody. However, as CL-K1 is a calcium-dependent C-type lectin, it is likely that the interference is due to calcium ions interacting with CL-LK. The structure of CL-K1 is unknown, but the crystal structures of other collectins, such as MBL and lung surfactant protein A and D, show that the CRDs of these collectins contain two calcium-binding sites (Weis et al., 1991; Sheriff et al., 1994; Hakansson et al., 1999; Head et al., 2003). Crystallographic studies performed at hyperphysiological concentrations of calcium revealed an artificial calcium-binding site in the CRD of MBL (Weis et al., 1992). It is likely that similar "extra" calcium ion binding sites exist in the CRD of CL-K1 and may cause conformational changes of the domain and disruption of an epitope in hyperphysiological concentrations of calcium.

Although we, to the best of our knowledge, are the first to report a comparison of calcium-sensitive and traditional immunoaffinity chromatographies and utilize calcium-sensitive immunoaffinity chromatography for the purification of a scarce plasma antigen, similar calcium-sensitive mAbs have been raised against the complement component C1r and factor VII and protein S and C of the coagulation system. These mAbs were successfully used for purification and to elucidate the conformational changes induced by calcium binding (Marcovina et al., 1989; Orthner et al., 1989; Coppola et al., 1992; Burgess and Thompson, 2002; Subramanian, 2002). Thus, generation of antibodies that only recognize the calcium-free conformation of a given protein seems to be an opportunity for many proteins and should be accounted for in the selection and cloning process of mAb-producing hybridomas. Many years of experience in the generation of mAbs against collectins in our department has shown us that immunization with antigens dissolved in buffers supplemented with EDTA results in high amount of calcium-sensitive or EDTA-dependent mAbs. Thus generation of such mAbs would only require minimal extra efforts and include a double screening process in the presence of EDTA or calcium and a verification that the selected mAb will dissociate from its antigen in the presence of calcium. By using aluminium hydroxide as adjuvant, which adsorbs and fixates the conformation of the antigen, it is likely that a calcium-free conformation of the antigen is preserved, although free calcium present in the tissue or blood surpasses the EDTA in the injected sample.

We found that calcium-sensitive immunoaffinity chromatography can be a superior method to traditional immunoaffinity chromatography. The technique may be applicable for the purification of many scarce plasma proteins using a single-step fractionation of plasma without the denaturation of eluted antigens. The technique may not only be applicable for traditional calcium-binding proteins but also for proteins that acquire and interact with calcium ions only at hyperphysiological calcium concentrations.

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References

Burgess, R.R., Thompson, N.E., 2002. Advances in gentle immunoaffinity chromatography. Curr. Opin. Biotechnol. 13, 304.

Coppola, R., Tombesi, S., Valentini, F., Alborali, S., Albertini, A., Mannucci, P.M., 1992. Characterization of a Ca (II)-independent monoclonal antibody that lacks reactivity with Gla-domainless human factor VII. Thromb. Res. 68, 269.

Hakansson, K., Lim, N.K., Hoppe, H.J., Reid, K.B., 1999. Crystal structure of the trimeric alpha-helical coiled-coil and the three lectin domains of human lung surfactant protein D. Structure 7, 255.

Hansen, S., Selman, L., Palaniyar, N., Ziegler, K., Brandt, J., Kliem, A., Jonasson, M., Skjoedt, M.O., Nielsen, O., Hartshorn, K., Jorgensen, T.J., Skjodt, K., Holmskov, U., 2010. Collectin 11 (CL-11, CL-K1) is a MASP-1/3-associated plasma collectin with microbial-binding activity. J. Immunol. 185, 6096.

Head, J.F., Mealy, T.R., McCormack, F.X., Seaton, B.A., 2003. Crystal structure of trimeric carbohydrate recognition and neck domains of surfactant protein A. J. Biol. Chem. 278, 43254.

Henriksen, M.L., Brandt, J., Andrieu, J.P., Nielsen, C., Jensen, P.H., Holmskov, U., Jorgensen, T.J., Palarasah, Y., Thielens, N.M., Hansen, S., 2013a. Heteromeric complexes of native collectin kidney 1 and collectin liver 1 are found in the circulation with MASPs and activate the complement system. J. Immunol. 191, 6117.

Henriksen, M.L., Brandt, J., Iyer, S.S., Thielens, N.M., Hansen, S., 2013b. Characterization of the interaction between collectin 11 (CL-11, CL-K1) and nucleic acids. Mol. Immunol. 56, 757.

Holmskov, U., Thiel, S., Jensenius, J.C., 2003. Collections and ficolins: humoral lectins of the innate immune defense. Annu. Rev. Immunol. 21, 547.

Keshi, H., Sakamoto, T., Kawai, T., Ohtani, K., Katoh, T., Jang, S.J., Motomura, W., Yoshizaki, T., Fukuda, M., Koyama, S., Fukuzawa, J., Fukuoh, A., Yoshida, I., Suzuki, Y., Wakamiya, N., 2006. Identification and characterization of a novel human collectin CL-K1. Microbiol. Immunol. 50, 1001.

Marcovina, S., Coppola, R., Valsecchi, C., Zoppo, A., Gelfi, C., Mannucci, P.M., 1989. Monoclonal antibodies directed to the calcium-free conformation of human protein S. Thromb. Haemost. 62, 708.

Moser, A.C., Hage, D.S., 2010. Immunoaffinity chromatography: an introduction to applications and recent developments. Bioanalysis 2, 769.

Nesterenko, M.V., Tilley, M., Upton, S.J., 1994. A simple modification of Blum's silver stain method allows for 30 minute detection of proteins in polyacrylamide gels. J. Biochem. Biophys. Methods 28, 239.

Ohtani, K., Suzuki, Y., Eda, S., Kawai, T., Kase, T., Yamazaki, H., Shimada, T., Keshi, H., Sakai, Y., Fukuoh, A., Sakamoto, T., Wakamiya, N., 1999. Molecular cloning of a novel human collectin from liver (CL-L1). J. Biol. Chem. 274, 13681.

Orthner, C.L., Madurawe, R.D., Velander, W.H., Drohan, W.N., Battey, F.D., Strickland, D.K., 1989. Conformational changes in an epitope localized to

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the NH2-terminal region of protein C. Evidence for interaction of protein C domains. J. Biol. Chem. 264, 18781.
Rhemrev-Boom, M.M., Yates, M., Rudolph, M., Raedts, M., 2001. (Immuno) affinity chromatography: a versatile tool for fast and selective purification, concentration, isolation and analysis. J. Pharm. Biomed. Anal. 24, 825.
Rooryck, C., Diaz-Font, A., Osborn, D.P., Chabchoub, E., Hernandez-Hernandez, V., Shamseldin, H., Kenny, J., Waters, A., Jenkins, D., Kaissi, A.A., Leal, G.F., Dallapiccola, B., Carnevale, F., Bitner-Glindzicz, M., Lees, M., Hennekam, R., Stanier, P., Burns, A.J., Peeters, H., Alkuraya, F.S., Beales, P.L., 2011. Mutations in lectin complement pathway genes COLEC11 and MASP1 cause 3MC syndrome. Nat. Genet. 43, 197.
Selman, L., Hansen, S., 2012. Structure and function of collectin liver 1 (CL-L1) and collectin 11 (CL-11, CL-K1). Immunobiology 217, 851.
Selman, L., Skjodt, K., Nielsen, O., Floridon, C., Holmskov, U., Hansen, S., 2008. Expression and tissue localization of collectin placenta 1 (CL-P1, SRCL) in human tissues. Mol. Immunol. 45, 3278.
Selman, L., Henriksen, M.L., Brandt, J., Palarasah, Y., Waters, A., Beales, P.L., Holmskov, U., Jorgensen, T.J., Nielsen, C., Skjodt, K., Hansen, S., 2012. An enzyme-linked immunosorbent assay (ELISA) for quantification of human collectin 11 (CL-11, CL-K1). J. Immunol. Methods 375, 182.
Sheng, S., Kong, F., 2012. Separation of antigens and antibodies by immuno-affinity chromatography. Pharm. Biol. 50, 1038.
Sheriff, S., Chang, C.Y., Ezekowitz, R.A., 1994. Human mannose-binding protein carbohydrate recognition domain trimerizes through a triple alpha-helical coiled-coil. Nat. Struct. Biol. 1, 789.
Subramanian, A., 2002. Immunoaffinity chromatography. Mol. Biotechnol. 20, 41.
Weis, W.I., Kahn, R., Fourme, R., Drickamer, K., Hendrickson, W.A., 1991. Structure of the calcium-dependent lectin domain from a rat mannose-binding protein determined by MAD phasing. Science 254, 1608.
Weis, W.I., Drickamer, K., Hendrickson, W.A., 1992. Structure of a C-type mannose-binding protein complexed with an oligosaccharide. Nature 360, 127.
Yoshizaki, T., Ohtani, K., Motomura, W., Jang, S.J., Mori, K., Kitamoto, N., Yoshida, I., Suzuki, Y., Wakamiya, N., 2012. Comparison of human blood concentrations of collectin kidney 1 and mannan-binding lectin. J. Biochem. 151, 57.

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