Journal of Visualized Experiments

Purification of human S100A12 and its ion-induced oligomers for immune cell stimulation

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE60065R1
Full Title:	Purification of human S100A12 and its ion-induced oligomers for immune cell stimulation
Keywords:	S100A12, DAMP, protein purification, chromatography, anion-exchange chromatography, hydrophobic-interaction chromatography, size exclusion chromatography, chemical crosslinking, monocyte stimulation
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Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Muenster, NRW, Germany

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Muenster, 09.05.2019

Revision JoVE60065R1

Dear Editors,

Thank you for evaluating our manuscript.

Based on all issues listed in your decision letter, we now submit a revision of our manuscript, providing requested/suggested changes in the text and figures.

As requested, we submit a detailed point-by-point reply letter, a “clean” copy of the revised paper as well as a “marked-up” version of the reviewed manuscript indicating revised parts in red font. Changes that refer to more “global” editorial comments and required i.e. re-writing of the papers abstract etc were introduced without extra highlighting.
Suggestions of protocol elements to be video-taped are highlighted in yellow.

We hope you will find the revised manuscript suitable for publication in *JoVE* and we look forward to your reply.

Sincerely,

Christoph Kessel

TITLE:

Purification of Human S100A12 and Its Ion-Induced Oligomers for Immune Cell Stimulation

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KEYWORDS:

S100A12, DAMP, protein purification, chromatography, anion-exchange chromatography, hydrophobic-interaction chromatography, size-exclusion chromatography, chemical crosslinking, monocyte stimulation

SUMMARY:

This protocol describes a purification method for recombinant tag-free calcium binding protein S100A12 and its ion-induced oligomers for human monocyte stimulation assays.

ABSTRACT:

In this protocol, we describe a method to purify human calcium-binding protein S100A12 and its ion-induced oligomers from *Escherichia coli* culture for immune cell stimulations. This protocol is based on a two-step chromatography strategy, which comprises protein pre-purification on an anion-exchange chromatography column and a subsequent polishing step on a hydrophobic-interaction column. This strategy produces S100A12 protein of high purity and yield at manageable costs. For functional assays on immune cells eventual remnant endotoxin contamination requires careful monitoring and further cleaning steps to obtain endotoxin-free protein. The majority of endotoxin contaminations can be excluded by anion-exchange chromatography. To deplete residual contaminations, this protocol describes a removal step with centrifugal filters. Depending on the available ion-strength S100A12 can arrange into different homomultimers. To investigate the relationship between structure and function, this protocol further describes ion-treatment of S100A12 protein followed by chemical crosslinking to stabilize S100A12 oligomers and their subsequent separation by size-exclusion chromatography. Finally, we describe a cell-based assay that confirms the biological activity of the purified protein and confirms LPS-free preparation.

INTRODUCTION:

S100A12 is a calcium binding protein which is predominantly produced by human granulocytes.

The protein is overexpressed during (systemic) inflammation and its serum levels, particularly in (auto)inflammatory diseases such as systemic juvenile idiopathic arthritis (sJIA), familial Mediterranean fever (FMF) or Kawasaki disease (KD) can inform about disease activity and response to therapy. Depending on pattern recognition receptors (PRRs) such as toll-like receptors (TLRs), the innate immune system can be activated by pathogen-associated molecular patterns (PAMPs) like lipopolysaccharides (LPS) or damage associated molecular patterns (DAMPs; also termed 'alarmins'). DAMPs are endogenous molecules such as cellular proteins, lipids or nucleic acids[1]. DAMP-functions are well described for the members of the calgranulin protein family, S100A8/A9 and S100A12[2], which are also reported to operate as divalent metal ion-chelating antimicrobial peptides[3-6]. Depending on the available ion strength S100A12 can, like other members of the S100 family, arrange into different homomultimers and until recently the impact of S100A12-oligomerisation on PRR-interaction, particularly TLR4, was unknown.

The protein's monomeric form (92 amino acids, 10.2 kDa) consists of two EF-hand helix-loop-helix structures connected by a flexible linker. The *C*-terminal EF-hand contains the classical Ca^{2+}-binding motif whereas the *N*-terminal EF-hand exhibits an S100 protein-specific extended loop structure ('pseudo-EF-hand') and reveals reduced Ca^{2+}-affinity. Ca^{2+}-binding by S100A12 can induce a major conformational change in the proteins' *C*-terminus, which results in exposure of a hydrophobic patch on each monomer and forms the dimerization interface. Thus, under physiological conditions, the smallest quaternary structure formed by S100A12 is a non-covalent dimer (approximately 21 kDa) in which individual monomers are in antiparallel orientation. When arranged as dimer, S100A12 is reported to sequester Zn^{2+} as well as other divalent metal ions, e.g., Cu^{2+} with high affinity[7]. These ions are coordinated at the S100A12 dimer interface by amino acids H15 and D25 of one subunit and H85 as well as H89 of the anti-paralleling other subunit[8-10]. While earlier studies propose that Zn^{2+}-loaded S100A12 may induce the protein's organization into homo-tetramers (44 kDa) and to result in increased Ca^{2+}-affinity[11,12], recent metal titration studies[6] suggest Ca^{2+}-binding by S100A12 to increase the protein's affinity to Zn^{2+}. Once the S100A12 EF-hands are fully occupied by Ca^{2+}, additional Ca^{2+} is thought to bind between dimers, triggering hexamer formation (approximately 63 kDa). The architecture of the hexameric quarternary structure is clearly different from that of the tetramer. It is proposed that the tetramer interface is disrupted to give rise to new dimer-dimer interfaces which benefits hexamer formation[10]. S100A12 is almost exclusively expressed by human granulocytes where it constitutes about 5% of all cytosolic protein[13]. In its DAMP function S100A12 was historically described as agonist of the multi-ligand receptor for advanced glycation end-products (RAGE), then termed extracellular newly identified RAGE-binding protein (EN-RAGE)[14]. Albeit we earlier reported biochemical S100A12-binding to both RAGE and TLR4[15], we recently demonstrated human monocytes to respond to S100A12 stimulation in a TLR4-dependent manner[16]. This requires arrangement of S100A12 into its Ca^{2+}/Zn^{2+}-induced hexameric quarternary structure[16].

Here we describe a purification procedure for recombinant human S100A12 and its ion-induced oligomers for immune cell stimulations[16,17]. This is based on a two-step chromatography strategy, which initially includes an anion-exchange column to isolate and concentrate the protein and remove bulk contaminations (e.g., endotoxins/lipopolysaccharides)[18]. Ion-exchange chromatography resins separate proteins on the basis of different net surface charges. For acidic

proteins like S100A12 (isoelectric point of 5.81), a buffer system with a pH of 8.5 and a strong anion-exchange resin leads to a good separation. Bound proteins were eluted with a high-salt buffer gradient. With an increase of ionic strength negative ions in the elution buffer compete with proteins for charges on the surface of the resin. Proteins individually elute depending on their net charge and in result of that, the buffers described herein allow to isolate and concentrate the overexpressed S100A12 protein. Due to negatively charged groups in lipopolysaccharides, these molecules also bind to anion-exchange resins. However, their higher net charge results in later elution in the applied high-salt gradient. The second step of the purification procedure has been introduced for polishing purposes. This makes use of the calcium binding ability of S100A12 and removes remaining impurities on a hydrophobic-interaction column. Calcium binding of S100A12 leads to a conformational change and an exposure of hydrophobic patches on the surface of the protein. On that condition, S100A12 interacts with the hydrophobic surface of the resin. Upon calcium-chelating by EDTA, this interaction is reversed. In the presence of ions, especially calcium and zinc, S100A12 arranges into homomeric oligomers. To study structure-function relationships of the different oligomers, we stabilized dimeric, tetrameric and hexameric recombinant S100A12 with a chemical crosslinker and separated the complexes on a size-exclusion chromatography column. Finally, to analyze functionality and biological activity of the purified protein and its ion-induced oligomers, the cytokine release of S100A12 and LPS stimulated monocyte can be compared.

Various methods for purifying S100A12 have been described so far. Jackson et al.[19], for example, published a protocol with purification via an anion-exchange column and a subsequent size-exclusion chromatography. Purification polishing on a size-exclusion column leads to good results, but—due to for example limited loading volumes—is less flexible in scalability. A different approach, published by Kiss et al.[20], describes purification of tagged protein via Ni^{2+} affinity column as the first purification step, followed by enzymatic cleavage to remove the tag and further purification steps. In contrast to the aforecited studies[19,20], the produced protein as described in this protocol is determined for experiments on immune cells. Therefore, remnant endotoxin contamination from bacterial culture is a challenge. Although different approaches for endotoxin removal have been described so far, there is no uniform method that works equally well for any given protein solution[21,22].

In summary, our protocol combines the advantages of a tag-free expression in a bacterial system with efficient endotoxin removal and high yield of pure protein.

PROTOCOL:

All patients' serum samples were collected at the University Children's Hospital, Muenster. All patients or parents provided written informed consent. The study was approved by the local ethics committee.

NOTE: Please refer to **Supplemental Table 1** for preparation of buffers and stock solutions.

1. Protein expression in *E. coli*

1.1. Cloning

1.1.1. Clone tag-free human S100A12 (NCBI Reference Sequence: NP_005612.1) into bacterial expression vector pET11b. To express the protein, transform the construct into *E. coli* BL21(DE3).

1.2. Culture

1.2.1. Prepare a starter culture by inoculating a single colony in 5 mL of growth medium (LB broth with 100 µg/mL ampicillin) in a 14 mL round-bottom tube. Incubate overnight at 37 °C with shaking at 220 rpm. Transfer 2−4 mL of overnight culture into 400 mL of growth medium in a 2 L Erlenmayer flask and incubate the culture at 37 °C with shaking at 220 rpm.

NOTE: Initial density of the main culture should be optical density at 600 nm (OD_{600}) = 0.1.

1.2.2. Monitor the OD_{600} during growth. Induce protein expression by addition of 1 M isopropyl-ß-D-thiogalactopyranosid (IPTG) to a final concentration of 1 mM at OD_{600} = 0.5−0.6. Incubate at 37 °C and 220 rpm for additional 4 h.

NOTE: In general, an OD_{600} of 0.6 will be reached after 1.5−2.5 h at 37 °C.

1.2.3. Prepare 50 mL sonication buffer by dissolving 50 mM Tris, 50 mM NaCl and 1 mM ethylenediamine tetraacetic acid (EDTA) in 40 mL of deionized water. Adjust pH with HCl to 8.0 and make up to 50 mL. Add protease inhibitor (1 tablet per 50 mL solution) and equilibrate the buffer to 4 °C.

1.2.4. Transfer the bacterial culture into suitable centrifuge bottles and harvest the cells at 3200 x *g* for 30 min at 4 °C. Discard the supernatant and resuspend the pellet in 25 mL of ice-cold sonication buffer. Henceforth keep the cells on ice.

NOTE: Resuspended cells can be stored at -20 °C for short-term and at -80 °C for long-term.

1.3. Sonication/lysis

1.3.1. Sonicate the cells for 6 cycles of 30 s on ice. After each cycle, rest cells for 30−60 s to protect the cells from overheating.

1.3.2. Transfer the cell suspension to a pre-chilled 50 mL high-speed centrifugation tube and centrifuge in a fixed angle rotor at 15,000 x *g* for 30 min at 4 °C. Decant the cleared lysate which contains the soluble cytosolic proteins into a fresh 50 mL tube and discard the pellet.

2. Protein purification

2.1. Anion-exchange chromatography

2.1.1. Dialysis

2.1.1.1. Prepare anion-exchange chromatography (AIEX) buffer A by dissolving 20 mM Tris, 1 mM EDTA and 1 mM ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) in deionized water and adjust the pH to 8.5 with HCl. For dialysis prepare 2 x 5 L and for chromatography 2 x 1 L of AIEX buffer A.

NOTE: The dialysate volume should be at about 100 times the sample volume. All buffers used for chromatography should be filtered (0.45 µm or smaller) and degassed (e.g., by ultrasonic bath or vacuum degassing).

2.1.1.2. Cut dialysis tubing (molecular weight cut-off [MWCO]: 3.5 kDa) into an appropriate length with additional space for air to ensure sample buoyancy above the rotating stir bar.

NOTE: Glycerol preserves the membrane and must be removed before use.

2.1.1.3. To reduce the viscosity of the cleared protein solution from step 1.3.2, dilute the solution with 25 mL of AIEX buffer A to facilitate subsequent application to the chromatography column. Attach the first closure onto the tubing, load the sample into the membrane and attach the second closure at least 3 cm from the top end of the tubing.

2.1.1.4. Place the 5 L container with AIEX buffer A on a stir plate, add a stir bar and the membrane filled with protein solution. Adjust the speed to rotate the sample by avoiding interference with the rotating stir-bar. Dialyze for 12−24 h at 4 °C, then replace the dialysate buffer (AIEX buffer A) by a fresh pre-cooled preparation and continue for at least 4 additional hours. Transfer the dialyzed protein solution to a 50 mL tube and filter through a 0.45 µm filter unit.

NOTE: Storage possible.

2.1.2. Chromatography

2.1.3. Start the liquid chromatography system (FPLC) with general maintenance, connect column buffers AIEX A and AIEX B (AIEX buffer A with 1 M NaCl) and the anion-exchange resin containing column. Refer to **Table 1** for general chromatographic parameters.

NOTE: Buffers, column and FPLC equipment should be equilibrated to the same temperature before starting the run (refer to chromatographic parameters in **Table 1**, **Table 2**, **Table 3**, **Table 4**, and **Table 5**).

2.1.4. Equilibrate the column with AIEX buffer A, subsequently load the sample onto the column and elute the proteins with a linear gradient from 0% to 100% high-salt buffer (AIEX B). Refer to **Table 2** for a detailed method protocol.

2.1.5. Collect 2 mL fractions during elution and analyze 10 µL of each fraction on a Coomassie-

stained 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Pool the fractions containing S100A12 protein for dialysis.

NOTE: The molecular weight of S100A12 is 10,575 Da.

2.2. Calcium-dependent hydrophobic-interaction chromatography (HIC)

2.2.1. Dialysis

2.2.1.1. Dialyze the protein solution against 20 mM Tris, 140 mM NaCl, pH 7.5 following the procedure described in section 2.1.1.

2.2.2. Chromatography

2.2.2.1. Prepare 1 L of chromatography buffer HIC A by dissolving 20 mM Tris, 140 mM NaCl and 25 mM $CaCl_2$ in deionized water and adjust the pH to 7.5. For HIC buffer B, dissolve 20 mM Tris, 140 mM NaCl and 50 mM EDTA. Adjust the pH to 7.0 and filter and degas the buffers. Add $CaCl_2$ to the sample to a final concentration of 25 mM and filter through 0.45 µm. Equilibrate HIC buffers and sample to 4 °C (column temperature).

2.2.2.2. Start the liquid chromatography system with general maintenance, connect column buffers HIC A and B and the column. Refer to **Table 3** for further chromatographic parameters.

2.2.2.3. Equilibrate the column, load the sample and extend the 'wash unbound sample' block until the UV signal reaches baseline level again. Then start elution with a calcium chelator containing buffer (EDTA). Refer to **Table 4** for a detailed method protocol.

NOTE: Previous experiments have shown that an excess of calcium seems to be beneficial for binding of S100A12 to the chromatography resin.

2.2.2.4. Collect peak fractions of 2 mL and analyze 10 µL of each fraction on a Coomassie-stained 15% SDS-PAGE. Pool pure S100A12 fractions and dialyze against Hepes-buffered saline (HBS; 20 mM Hepes, 140 mM NaCl, pH 7.0) as described in section 2.1.1.

NOTE: Extinction coefficient of monomeric S100A12 is 2980 M^{-1} cm^{-1}.

3. Detection and removal of endotoxin

3.1. Detection of endotoxin

3.1.1. To determine the endotoxin contamination, measure concentrations of diluted protein from step 2.2.2.4. (e.g., 1:5 and 1:10 in HBS) using an enzyme-linked immunosorbent assay (ELISA)-based, fluorescent endotoxin detection assay (**Table of Materials**). Perform this assay by following the manufacturer's protocol.

NOTE: Use freshly prepared HBS solutions dissolved in ultrapure deionized water to avoid (new) endotoxin contamination by the buffer.

3.2. Removal of endotoxin and concentration of protein

3.2.1. Load 15 mL of sample onto a 50 kDa centrifugal filter unit and centrifuge at 3200 x *g* and 10 °C for approximately 10 min. Transfer the flow-through into a fresh vessel (on ice) and refill and centrifuge the 50 kDa filter tube as often as necessary. Wash the filter membrane twice with HBS to recover as much protein as possible after each step.

3.2.2. Concentrate the S100A12-containing flow-through by using a 3 kDa centrifugal filter until the volume is reduced to one fifth up to one tenth of the initial loading volume (centrifugation at 3200 x *g*, 10 °C for approximately 30 min). Refill the filter as often as necessary, rinse the membrane and transfer the concentrated solution to a new tube after each refill. Discard the flow-through. Filter again through 50 kDa as described above.

NOTE: During this procedure, the loss of protein is remarkable (up to 50%), but the remaining protein preparation is completely depleted from LPS. This method yields about 10−15 mg protein from 400 mL culture.

3.2.3. Adjust the protein solution to 1 mg/mL with endotoxin-free HBS and measure the LPS content as described in step 3.1.1. In case the protein solution is still not tested as LPS-free (< 0.1 EU/mL), eliminate remnant contaminations by using an endotoxin removal resin.

NOTE: With a protein concentration of 1 mg/mL, contamination of 0.1 EU/mL LPS equals approximately 0.01 pg LPS/µg protein.

4. Chemical crosslinking and oligomer separation

4.1. Chemical crosslinking

4.1.1. Prepare highly pure (endotoxin-free) stock solutions of 1 M $CaCl_2$ and 100 mM $ZnCl_2$ in ultrapure deionized water (**Table of Materials**). Use this buffer, freshly made, for the next step.

4.1.2. Incubate 10 mL of purified endotoxin-free S100A12 (concentration 1 mg/mL in HBS) for 30 min at room temperature (RT) with either 25 mM $CaCl_2$ for dimeric/tetrameric, or 25 mM $CaCl_2$ and 1 mM $ZnCl_2$ for hexameric/tetrameric S100A12 oligomers.

4.1.3. Prepare crosslinker by dissolving 8 mg of BS^3 in 500 µL of endotoxin-free water directly before use (8 mg crosslinker for 10 mL ion-spiked protein solution equals a final concentration of 1.4 mM). Mix crosslinker and sample by pipetting and incubate for additional 30 min at RT. Quench the reaction by adding 1 M Tris-HCl, pH 7.5 to a final concentration of 50 mM and filter through 0.45 µm.

309
310 4.2. Size-exclusion chromatography
311
312 4.2.1. Equilibrate the crosslinked sample to 12−15 °C (column temperature) and start the liquid
313 chromatography system with general maintenance. Connect column buffer (HBS) and the size-
314 exclusion column. Refer to **Table 5** for detailed information.
315
316 4.2.2. Equilibrate the column in HBS, load sample and collect peak fractions (1−2 mL) during the
317 run. Analyze these fractions on a 4−20% gradient SDS-PAGE and pool fractions with major bands
318 of the desired protein complex.
319
320 NOTE: Hydrolysis of NHS ester reagents like BS^3 in aqueous solutions results in a strong
321 absorbance at 280 nm. Unbound crosslinker (molecular weight: 572 g/mol) elutes at the end of
322 the run and results in a strong peak.
323
324 4.2.3. Concentrate the solutions by using centrifugal filter units with MWCOs of 10 kDa (dimer),
325 30 kDa (tetramer) or 50 kDa (hexamer). Determine the endotoxin contamination as described in
326 section 3.1. If necessary, remove remaining LPS with an endotoxin removal resin following the
327 manufacturer's recommendations (**Table of Materials**).
328
329 **5. Functional testing on monocytes**
330
331 5.1. Preparation of monocytes
332
333 5.1.1. Isolate monocytes from human buffy coats by density gradient centrifugation and
334 subsequent monocyte enrichment by using a magnetic bead separation kit (**Table of Materials**).
335
336 NOTE: This protocol will result in approximately 5−7 x 10^7 monocytes (one buffy coat) with a
337 purity of 83−95%. Since the number, but also the responsiveness of cells depends strongly on the
338 donor, the protocol may have to be scaled up (depending on the required cell count).
339
340 5.1.2. For density centrifugation, equilibrate the separation solution (density = 1.077 g/mL) to RT
341 and transfer 20 mL into 50 mL centrifuge tubes (2 tubes per buffy coat). Dilute blood from the
342 human buffy coat with Hank's buffered salt solution (HBSS) to a total volume of 60 mL and layer
343 30 mL of this mixture carefully on top of the separation medium. Centrifuge at 550 x *g* for 35 min
344 at RT. Disable the centrifuge brake.
345
346 5.1.3. After centrifugation, the mononuclear peripheral blood cells (PBMCs) are located directly
347 on top of the separation medium. Transfer these cells into a fresh 50 mL centrifuge tube, make
348 up to 50 mL with HBSS, and centrifuge at 170 x *g* for 10 min. Aspirate the supernatant and
349 resuspend the cell pellet in a small volume of HBSS by pipetting.
350
351 5.1.4. Fill the tube up to 50 mL and centrifuge at 290 x *g* for 10 min without brake. Aspirate the
352 supernatant again, resuspend the cells in HBSS (50 mL) and centrifuge at 170 x *g* for 10 min. Count

the cells and resuspend them in cell separation buffer (**Table of Materials**) to a concentration of 5 x 10^7 cells/mL.

NOTE: Instead of HBSS, phosphate-buffered saline (PBS) can be used for washing the cells.

5.1.5. For monocyte isolation from PBMCs, use a magnetic negative cell isolation kit and follow the manufacturer's protocol. Count monocytes and resuspend in monocyte medium (RPMI 1640, 15% heat-inactivated fetal calf serum [FCS], 4 mM L-glutamine, 100 U/mL penicillin/streptomycin) to a concentration of 2 x 10^6 cells/mL.

5.1.6. To culture monocytes, coat culture dishes (e.g., 100 mm) with a hydrophobic, gas-permeable film, suitable for suspension cells (**Table of Materials**). Sterilize the plates by using UV light for approximately 30 min. Transfer the cells to these culture plates and let them rest over-night at 37 °C and 5% CO_2.

NOTE: Use 15−25 mL of cell suspension per coated dish.

5.2. Monocyte stimulation

5.2.1. Stimulation with S100A12 (wildtype)

NOTE: To distinguish untreated S100A12 (end-product from section 2.2.2) from crosslinked protein, S100A12 in the following is referred to as 'wildtype'.

5.2.1.1. Transfer the rested cells into a 50 mL centrifugal tube and centrifuge at 350 x *g* for 10 min. Aspirate the supernatant and resuspend the cell pellet in stimulation medium (RPMI 1640, 5% heat-inactivated FCS, 4 mM L-glutamine, 100 U/mL penicillin/streptomycin) at a concentration of 2 x 10^6 cells/mL.

5.2.1.2. For stimulation, use 24 well suspension plates and add 250 µL of cell suspension per well (0.5 x 10^6 cells/well). Add 50 µg/mL polymyxin B to the intended wells, followed by either LPS in different concentrations (25, 50, 100 and 200 pg/mL) or wildtype S100A12 (10, 20, 40, 60 µg/mL). Further, apply the protein either untreated or heat-denatured (99 °C, 10 min) in different concentrations to the cells.

NOTE: A short heat treatment denatures S100A12 protein but has less to no effect on LPS.

5.2.1.3. Incubate plates for 4 h at 37 °C and 5% CO_2. Harvest the cells by transferring the cell suspension of each well to 1.5 mL reaction tubes. Centrifuge at 500 x *g* for 10 min. Transfer the supernatants to fresh tubes and measure TNFα release in different dilutions (e.g., 1:2, 1:5, 1:10) with a human TNFα ELISA kit following the manufacturer's recommendations.

5.2.2. Stimulation with S100A12 oligomers

5.2.2.1. Prepare and seed out monocytes in 24 well suspension plates as described above. Stimulate cells by adding S100A12 oligomers from step 4.2.3. in different molar concentrations (125 nM, 250 nM, 500 nM, 1000 nM).

NOTE: In order to compare the abilities of the different oligomers to stimulate monocytes, oligomers were applied to the cells in comparable molar concentrations.

5.2.2.2. Incubate for 4 h at 37 °C and 5% CO_2, harvest the cells and measure TNFα release in the supernatants as described above.

REPRESENTATIVE RESULTS:

Following pre-purification on the AIEX column (**Figure 1A-C**) and subsequent calcium-dependent HIC (**Figure 2A,B**), highly pure protein was obtained (**Figure 2C**). In addition, measurements of endotoxin revealed successful LPS removal. The LPS content following AIEX was measured in a 1:10 dilution above the assay detection limit, i.e., above 500 EU/mL. After the first filtration through a 50 kDa filter unit, the LPS content was reduced to 1 EU/mL. Following concentration with a 3 kDa filter unit and additional filtration through 50 kDa, the measured LPS contamination was 0.08 EU/mL.

As an additional control, human monocytes were stimulated with the produced wildtype protein (**Figure 3A,B**). Polymyxin B treatment abrogates TNFα release from LPS-stimulated monocytes, which cannot be observed with S100A12. On the other hand, heat-treatment of both LPS and S100A12 abolishes the protein's capacity to stimulate cells, while this does not affect cellular response to LPS-stimulation.

Protein exposure to different ions results in arrangement of different S100A12 oligomers (**Figure 4A**). Chemical crosslinking allows to capture defined complexes such as dimers, tetramers, and hexamers as well as transition states (e.g., 'trimers', band at approximately 30 kDa). In order to induce a pronounced shift of the oligomer-equilibrium prior to crosslinking, an excess of ions was applied (**Figure 4B**).

Isolated oligomers in equal molar concentrations (**Figure 5A-C**) were then used for monocyte stimulation to compare signaling abilities via PRRs. Monocyte-stimulation with hexameric S100A12 resulted in pronounced TNFα release (**Figure 6**). Remnant cytokine release could be detected from cells stimulated with tetrameric S100A12, while treatment with dimeric protein does not induce TNFα release.

FIGURE AND TABLE LEGENDS:

Figure 1: Results of anion-exchange chromatography. (**A**) A chromatogram with absorbance at 280 nm (A_{280}) and the percentage of elution buffer B (dashed line). Methods blocks are indicated with A = wash unbound sample, B = linear gradient with elution buffer (buffer B), C = wash out with buffer B, and D = re-equilibration in buffer A. (**B**) Focus on the relevant peaks with fraction tube numbers in red. (**C**) Selected fractions were analyzed on 15% Coomassie-stained SDS-PAGE.

Figure 2: Results of hydrophobic-interaction chromatography. (**A**) A chromatogram with absorbance at 280 nm (A_{280}) and the percentage of elution buffer B (dashed line). Methods blocks are indicated with A = wash unbound sample, B = elution with buffer B, and C = re-equilibration in buffer A. (**B**) Focus on the relevant peaks with fraction tube numbers in red. (**C**) Analyzed fractions on 15% Coomassie-stained SDS-PAGE.

Figure 3: Primary human monocytes were stimulated at indicated concentrations. LPS (**A**) or S100A12 (wildtype, **B**) were left untreated or heat-denaturated (99 °C, 10 min). Both conditions were tested in the presence and absence of polymyxin B.

Figure 4: S100A12 protein was crosslinked with BS^3 after incubation in HBS buffer containing 5 mM Ca^{2+} and indicated Zn^{2+} concentrations. (**A**) Increasing Zn^{2+} concentrations induce arrangement of S100A12 into tetramers and hexamers upon separation on 4−20% Coomassie-stained SDS-PAGE. (**B**) Representative result of crosslinked oligomers with conditions as used for separation on a size-exclusion column. S100A12 was crosslinked in presence of either 25 mM Ca^{2+} (lane 1) or 25 mM Ca^{2+} and 1 mM Zn^{2+} (lane 2). $(S100A12)_2$ = dimer; $(S100A12)_4$ = tetramer; $(S100A12)_6$ = hexamer.

Figure 5: S100A12 oligomers were separated on a size-exclusion column. (**A**) Chromatogram of hexamer/tetramer separation after crosslinking in HBS buffer with 25 mM $CaCl_2$ and 1 mM $ZnCl_2$. (**B**) Chromatogram for tetramer/dimer separation in HBS buffer with 25 mM $CaCl_2$. (**C**) Example of pooled and concentrated oligomers after separation on a Coomassie-stained 4−15% gradient SDS-PAGE. Lane 1: dimer; lane 2: tetramer; lane 3: hexamer.

Figure 6: Stimulation of monocytes with purified S100A12 oligomers. TNFα-release after 4 h incubation was quantified by ELISA. The data show the mean value from two independent experiments.

Table 1: Detailed information on the applied parameters of anion-exchange chromatography.

Table 2: Detailed information on the used method of anion-exchange chromatography.

Table 3: Detailed information on the applied parameters of hydrophobic-interaction chromatography.

Table 4: Detailed information on the used method of hydrophobic-interaction chromatography.

Table 5: Detailed information for the applied parameters of size-exclusion chromatography.

Supplemental Table 1: Preparation of buffers and stock solutions.

DISCUSSION:

In this protocol, we describe tag-free bacterial expression of human S100A12 and its purification as well as separation into different ion-induced oligomers for immune cell stimulation. Compared to published literature on S100A12 protein purification[8,23,24], the use of high $CaCl_2$ (25 mM) in hydrophobic-interaction chromatography is to our knowledge unique. Several protocols applying concentrations from 1 to 5 mM do produce pure protein, yet we observed a several times higher yield following our approach using 25 mM $CaCl_2$ instead. This might be explained by a hierarchy of protein interaction with the column material:S100A12 can directly bind to the column material but the excess of Ca^{2+} may also facilitate indirect binding of S100A12-dimers to the already column-bound protein[8]. Thus, high Ca^{2+} concentrations may enlarge the surface available for S100A12 purification. Elution (by using a linear gradient) of S100A12 from HIC as one early (indirectly bound S100A12) and one very late peak (column material bound protein) may support this speculation (data not shown).

For production of recombinant S100A12 (as well as other proteins) at high yields and manageable costs, protein expression in *E. coli* is still the method of choice. However, the inevitable contamination with bacterial endotoxins remains a problem, when proteins are determined for cell culture experiments, particularly in studies involving innate immune cells. To our experience, even commercially available proteins explicitly declared for cell culture use can contain endotoxin contaminations up to 1 EU/µg protein, which can significantly skew assays. Therefore, a complete removal of endotoxins is mandatory. Endotoxin monomers in solution range from molecular weights of 10 to 20 kDa, but they can form micelles and structures with higher molecular weights. The formation of very large structures is, for example, promoted through bivalent ions[21,25].

According to our protocol, we verify the endotoxin-free production of S100A12 protein by combining high-sensitivity endotoxin measurements with monocyte stimulation assays. We consider such combination particularly meaningful as a) low-level endotoxin contamination may be difficult to assess depending on the sensitivity of the assay and b) the use of polymyxin B as LPS inhibitor on monocytes may result in difficult to interpret data due to exclusive polymyxin B effects on cells[26,27]. Polymyxin B as well as other cationic peptides are reported to bind LPS via negatively charged lipid A[28]. As the solvent exposed surface of S100A12 also contains large negatively charged patches the observed reduction of TNFα-release from S100A12-stimulated human monocytes in presence of polymyxin B (**Figure 3B**) may be due to a) unspecific direct binding of polymyxin B to S100A12 and/or b) direct effects of polymyxin B on stimulated cells[26,27]. Due to the known limitations of both the detection of low-level endotoxin contamination as well as unspecific polymyxin B effects, our protocol further contains a heat-inactivation step to clearly distinguish between LPS- and protein-mediated TLR4-signaling.

Use of LPS-free S100A12 for generation and purification of defined ion-induced oligomers is critical and extra attention should be paid to their subsequent purification to avoid eventual re-introduction of endotoxin via buffers or column material and thus further protein-demanding LPS-depletion via endotoxin removal resins.

The relevance of oligomerization for the biological function of proteins can be assessed by different means. In case of S100A12, we used surface plasmon resonance as well as targeted

amino acid exchanges at ion-binding sites and—to most precisely define the protein-complex able to bind and signal through TLR4—we employed chemical crosslinking of Ca^{2+}/Zn^{2+}-pulsed recombinant S100A12[16]. Chemical crosslinking of S100A12 under different ionic conditions snap-freezes a momentary state including several oligomeric forms that are in transition. From ion titration experiments, we defined conditions under which dimeric, tetrameric or hexameric oligomers could be determined as the predominant oligomers[16]. In addition, previous experiments have shown that an excess of ions is beneficial for comparable, stable crosslinking and subsequent purification, although oligomerization can also be induced at significantly lower ion concentrations. However, purifying these oligomers by size-exclusion chromatography results in good, but not absolute separation. Still, the selective enrichment of oligomers allows for reliable downstream analyses.

In summary, this protocol provides a method for purification of LPS-free human S100A12 or related calcium binding proteins. To fix ion-induced conformational changes, chemical crosslinking and subsequent complex separation by size-exclusion chromatography is a useful tool to understand the relevance of protein oligomerization for downstream biological processes.

ACKNOWLEDGMENTS:

This study was supported by grants from the intramural innovative medical research program of Muenster University medical faculty (KE121201 to C.K.) and the German Research Foundation (DFG, Fo354/3-1 to D.F.)**.**

DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

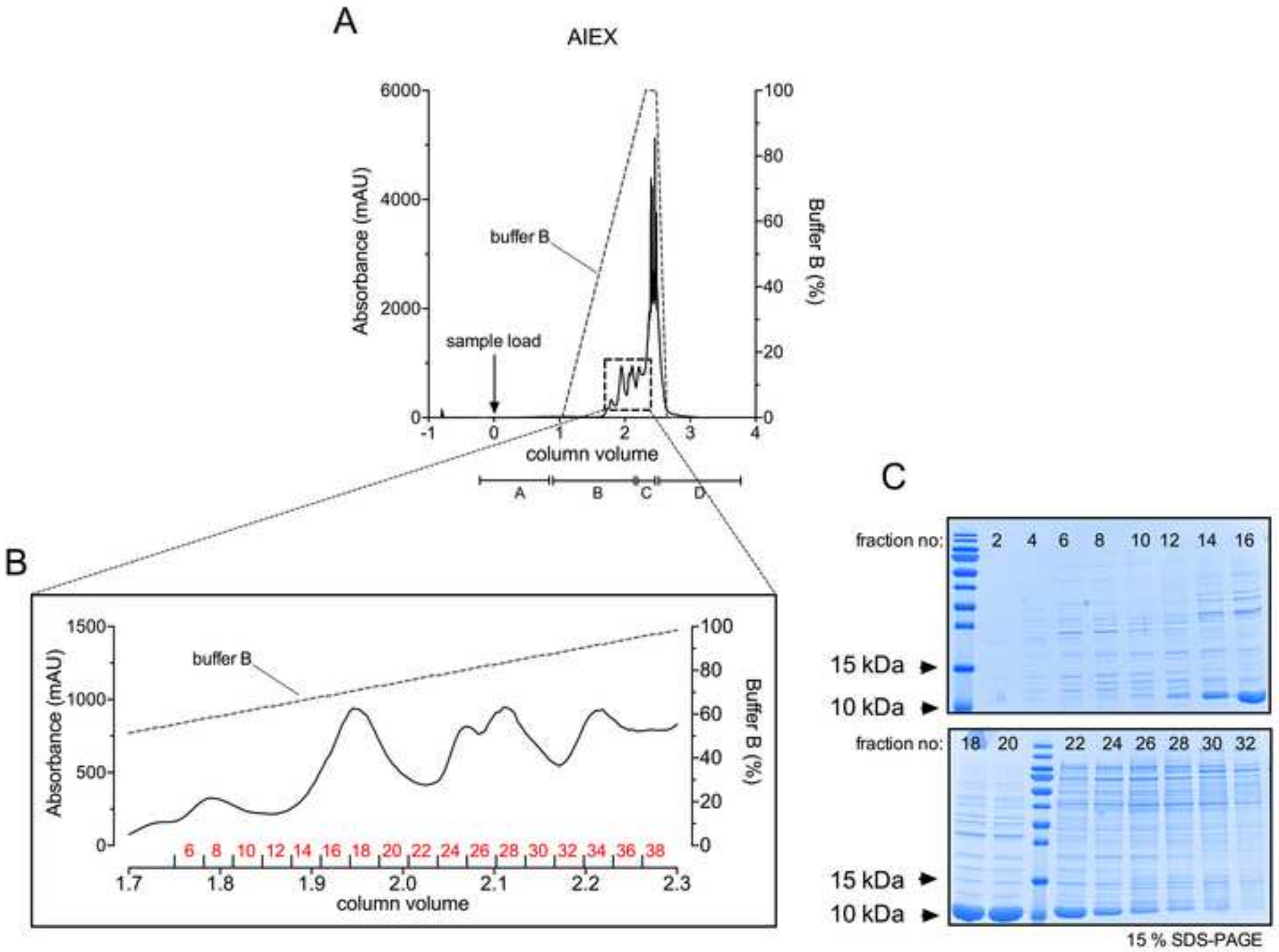

Figure 2

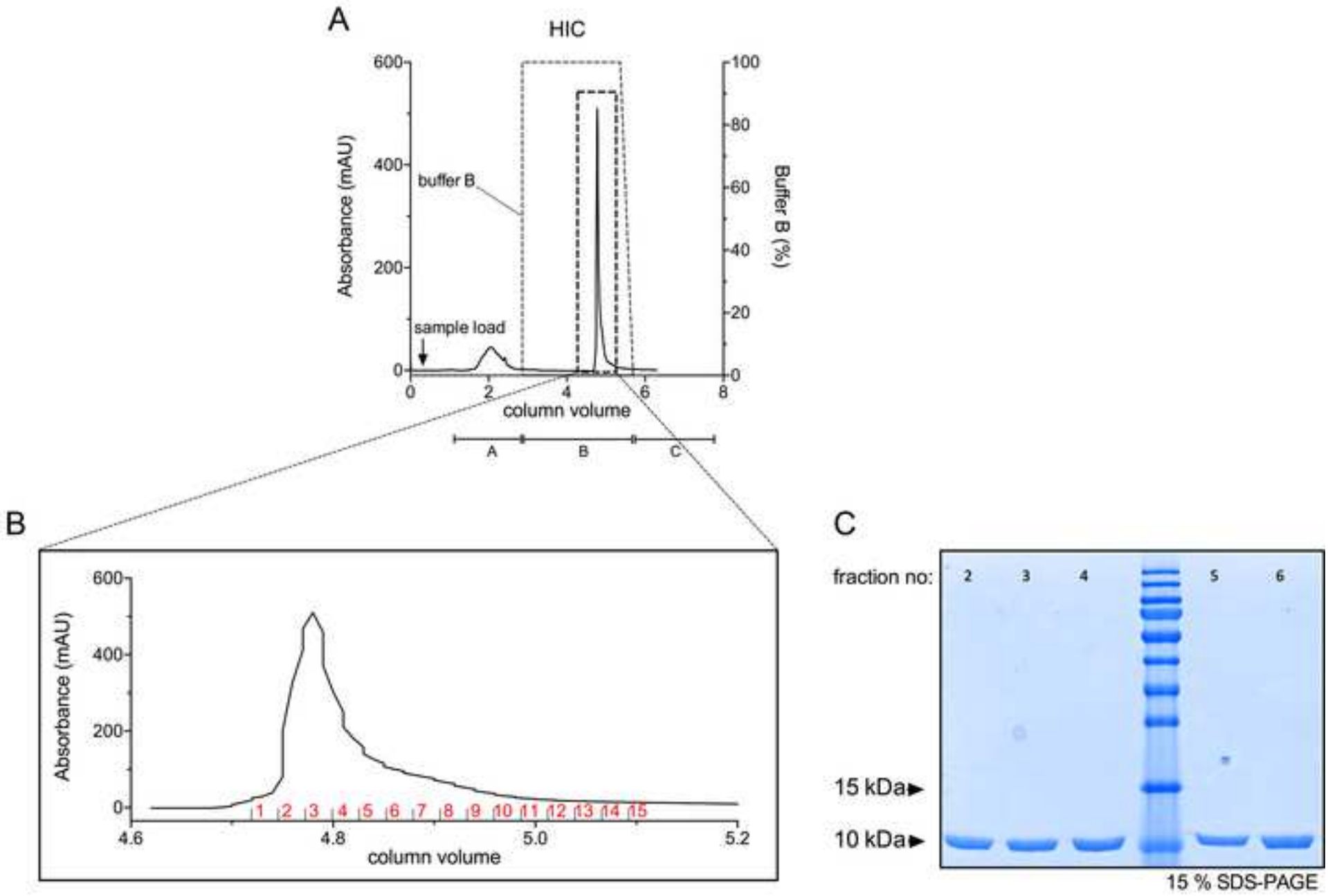

Figure 3

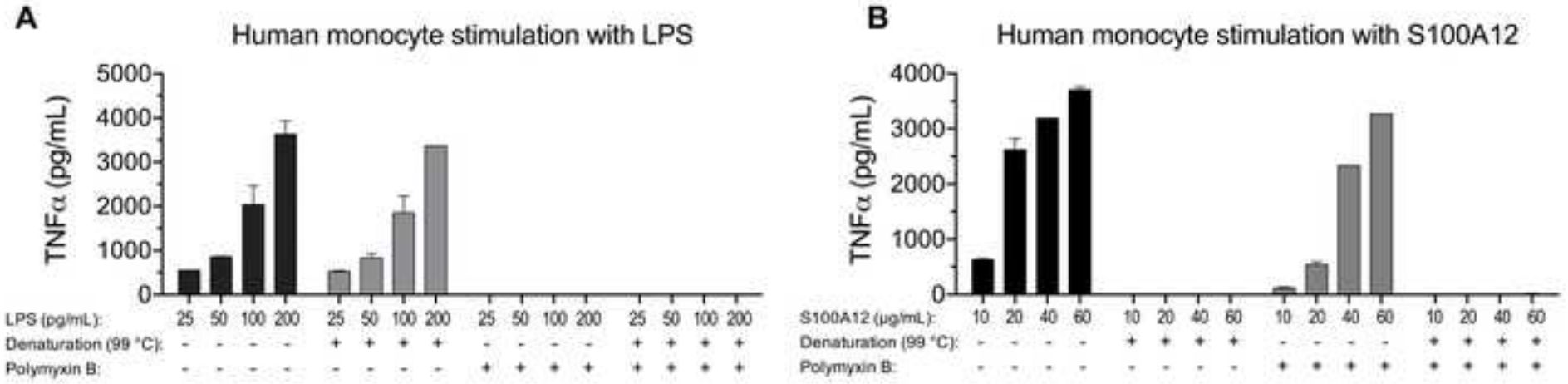

Figure 4

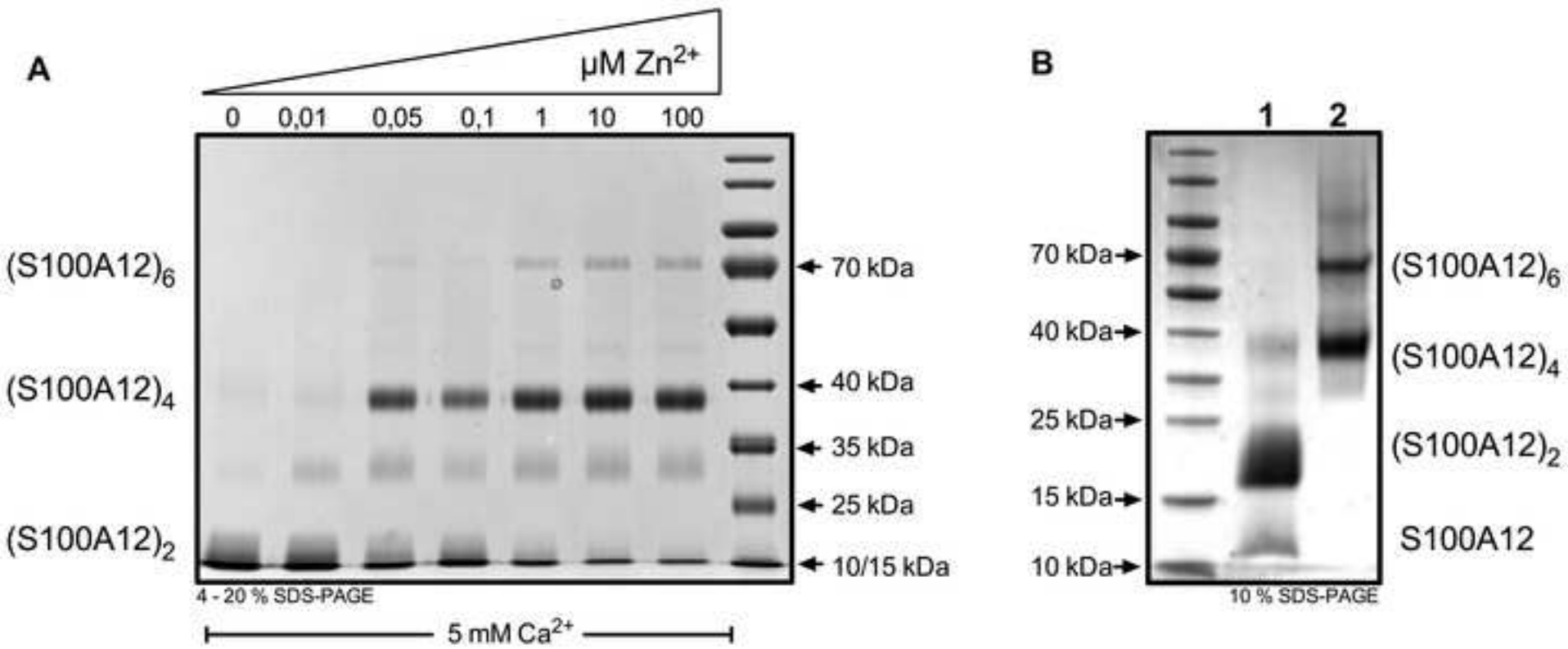

Figure 5

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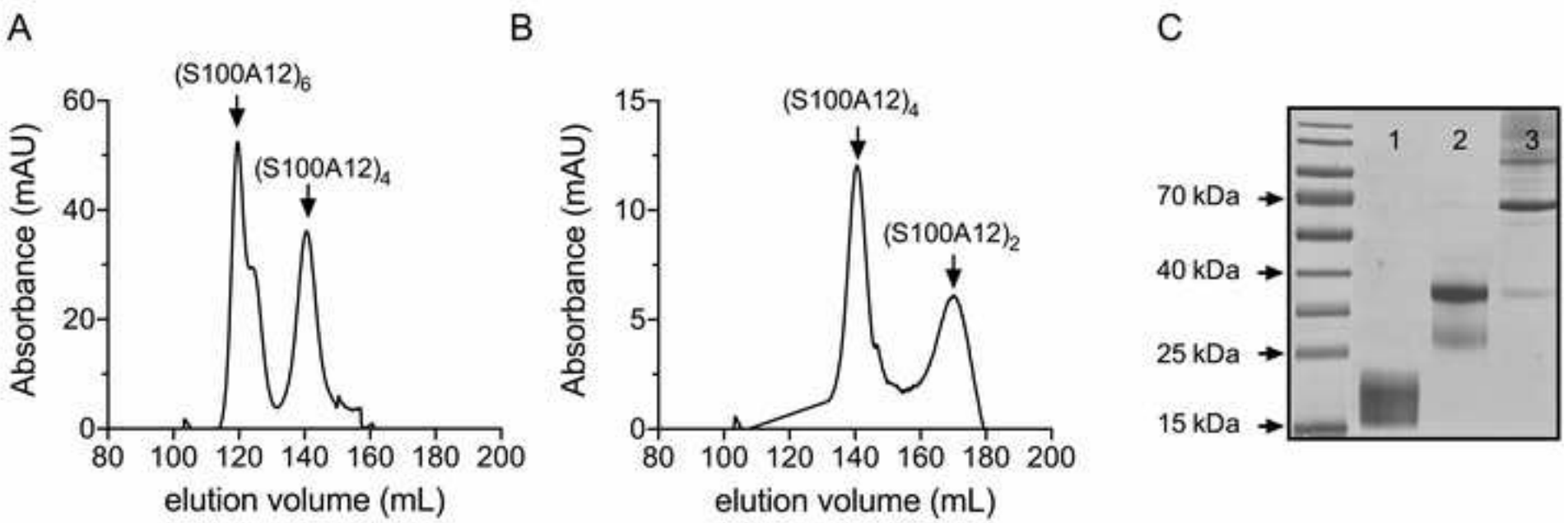

Figure 6

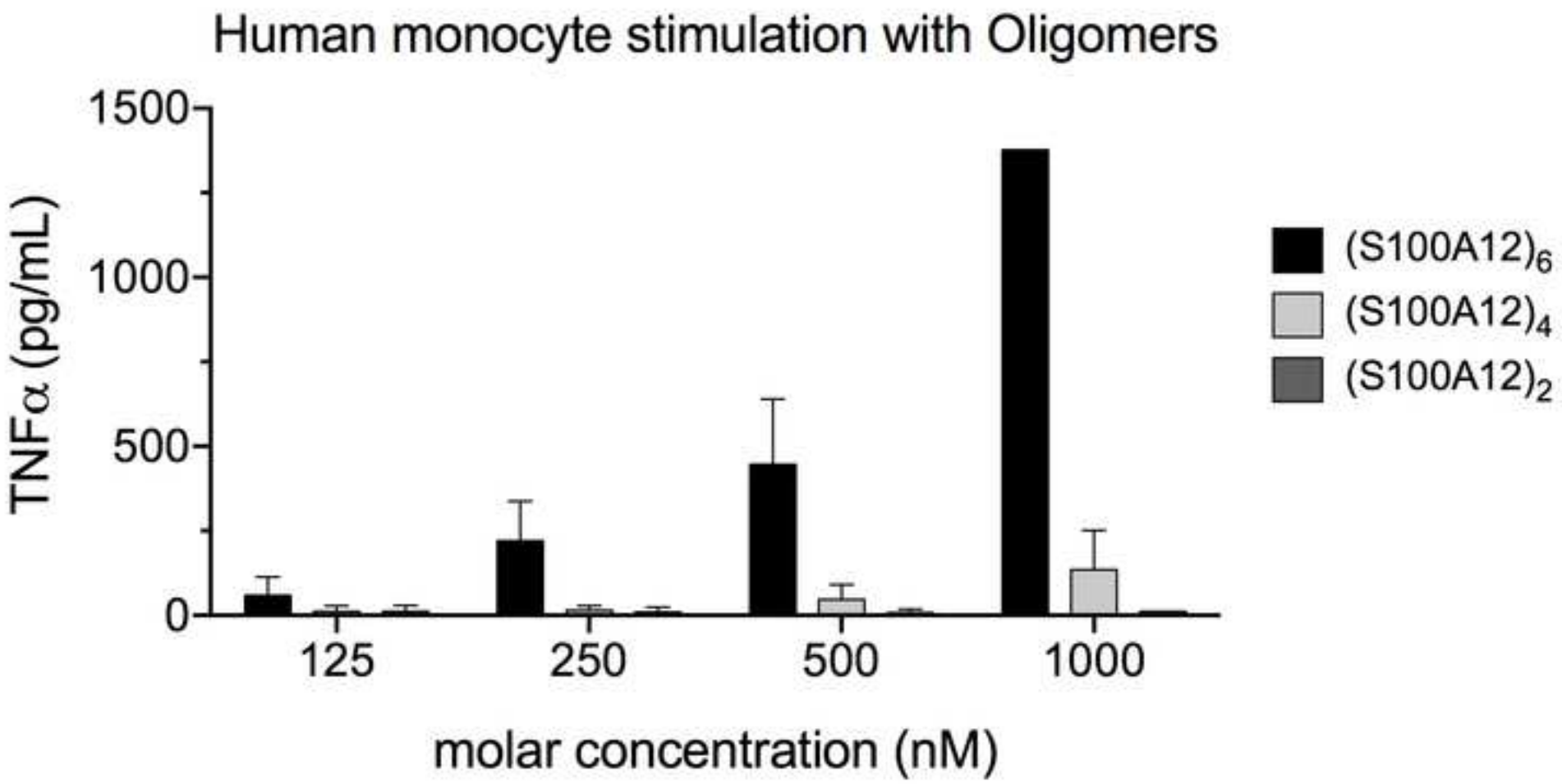

Table 1

Bed Volume (CV)	75 mL
Monitor	Absorbance at 280 nM
Pressure Max	3 bar
Column buffer A	20 mM Tris-HCl, 1 mM EDTA, 1 mM EGTA, pH 8.5
Column buffer B	20 mM Tris-HCl, 1 mM EDTA, 1 mM EGTA, 1 M NaCl, pH 8.5
Sample Volume	variable
Flow Rate	1−2 mL/min
Temperature	4 °C

Table 2

Block	Volume	Buffer	Outlet
Equilibration	1−2 column volumes (CVs)	A	Waste
Sample load	n/a	A	Waste
Wash out unbound sample	1 CV	A	High volume outlet
Gradient–Elution	0−100 % Buffer B in 1 CV	A to B	Fraction collector
Wash out–Buffer B	1 CV	B	Waste
Re-Equilibration	2 CVs	A	Waste

Table 3

Bed Volume (CV)	125 mL
Monitor	Absorbance at 280 nM
Pressure Max	4 bar
Column buffer A	20 mM Tris, 140 mM NaCl, 25 mM $CaCl_2$, pH 7.5
Column buffer B	20 mM Tris, 140 mM NaCl, 50 mM EDTA, pH 7.5
Sample Volume	variable
Flow Rate	1−2 mL/min
Temperature	4 °C

Table 4

Block	Volume	Buffer	Outlet
Equilibration	1–2 column volumes (CVs)	A	Waste
Sample load	n/a	A	Waste
Wash out unbound sample	1–2 CVs	A	High volume outlet
Gradient–Elution	0–100 % Buffer B in 1 CV	A to B	Fraction collector
Wash out–Buffer B	1 CV	B	Waste
Re-Equilibration	2 CVs	A	Waste

Table 5

Bed Volume (CV)	320 mL
Monitor	Absorbance at 280 nm
Pressure Max	3 bar
Column buffer A	20 mM Hepes, 140 mM NaCl, pH 7.2
Sample Volume	Up to 13 mL
Flow Rate	1−1.5 mL/min
Temperature	12−15 °C

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
pET11b vector	Novagen		
BL21(DE3) competent *E. coli*	New England Biolabs	C2527	
100 x Non-essential amino acids	Merck	K 0293	
25% HCl	Carl Roth	X897.1	
4−20% Mini-PROTEAN TGX Protein Gels	BioRad	4561093	
Ampicillin sodium salt	Carl Roth	HP62.1	
BS3 (bis(sulfosuccinimidyl)suberate) - 50 mg	ThermoFisher Scientific	21580	
Calciumchlorid Dihydrat	Carl Roth	5239.1	
Coomassie Briliant Blue R250 Destaining Solution	BioRad	1610438	
Coomassie Briliant Blue R250 Staining Solution	BioRad	1610436	
EasySep Human Monocyte Enrichment Kit	Stemcell	19059	Magnetic negative cell isolation kit
EDTA disodium salt dihydrate	Carl Roth	8043.1	
EGTA	Carl Roth	3054.3	
EndoLISA	Hyglos	609033	Endotoxin detection assay
Endotoxin-Free Ultra Pure Water	Sigma-Aldrich	TMS-011-A	Ultrapure water for preparation of endotoxin-free buffers
EndoTrap red	Hyglos	321063	Endotoxin removal resin
FBS (heat-inactivated)	Gibco	10270	
HBSS, no calcium, no magnesium	ThermoFisher Scientific	14175053	
Hepes	Carl Roth	9105	
Hepes (high quality, endotoxin testet)	Sigma-Aldrich	H4034	
hTNF-alpha - OptEia ELISA Set	BD	555212	
IPTG (isopropyl-ß-D-thiogalactopyranosid)	Carl Roth	CN08.1	
L-Glutamine (200 mM)	Merck	K 0282	
LB-Medium	Carl Roth	X968.1	
Lipopolysaccharides from *E. coli* O55:B5	Merck	L6529	
Pancoll, human	PAN Biotech	P04-60500	Separation solution (density gradient centrifugation)
Penicillin/Streptomycin (10.000 U/ml)	Merck	A 2212	
Phenyl Sepharose High Performance	GE Healthcare	17-1082-01	Resin for hydrophobic interaction chromatography
Polymyxin B	Invivogen	tlrl-pmb	
Protease inhibitor tablets	Roche	11873580001	
Q Sepharose Fast Flow	GE Healthcare	17-0510-01	Resin for anion-exchange chromatography
RoboSep buffer	Stemcell	20104	Cell separation buffer (section 5.1.4)
RPMI 1640 Medium	Merck	F 1215	
Sodium chloride (NaCl)	Carl Roth	3957.2	
Sodium hydroxide	Carl Roth	P031.1	
Tris Base	Carl Roth	4855.3	
Zinc chloride	Carl Roth	T887	

Labware

0,45 µm syringe filter	Merck	SLHA033SS	
14 mL roundbottom tubes	BD	352059	
2 L Erlenmyer flask	Carl Roth	LY98.1	
24 well suspension plates	Greiner	662102	
5 L measuring beaker	Carl Roth	CKN3.1	
50 mL conical centrifuge tubes	Corning	430829	
50 mL high-speed centrifuge tubes	Eppendorf	30,122,178	
Amicon Ultra-15 Centrifugal Filter Unit MWCO 3 kDa	Merck	UFC900324	
Amicon Ultra-15 Centrifugal Filter Unit MWCO 50 kDa	Merck	UFC905024	
Culture dish (100 mm)	Sarstedt	83.3902	
Dialysis Tubing Closures	Spectrum	132738	
EasySep magnet 'The Big Easy`	Stemcell	18001	
Fraction collector tubes 5 mL	Greiner	115101	
Lumox film, 25 µm, 305 mm x 40 m	Sarstedt	946,077,316	Film for monocyte culture plates
Spectra/Por Dialysis Membrane (3.5 kDa)	Spectrum	132724	
Steritop filter unit	Merck	SCGPT01RE	

Equipment

37 °C Incubator (with shaking)	New Brunswick Scientific	Innova 42	
ÄKTA purifier UPC 10	GE Healthcare		FPLC System
Fraction collector	GE Healthcare	Frac-920	
Centrifuge (with rotor A-4-81)	Eppendorf	5810R	
Fixed angle rotor	Eppendorf	F-34-6-38	
Mini Protean Tetra Cell	BioRad	1658000EDU	
NanoPhotometer	Implen	P330	
Sonicator	Brandelin	UW2070	
Fluorescence reader	Tecan	infinite M200PRO	
pH meter	Knick	765	

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Editor:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Autor reply: We have proof read the manuscript and corrected remnant spelling/grammar issues.

2. Abstract: Please revise to include a statement about the purpose of the method, an overview of the method and a summary of its advantages, limitations, and applications.

Autor reply: We rewrote the abstract to meet this point.

3. Introduction: Please expand to include the advantages of the presented method over alternative techniques with applicable references to previous studies.

Autor reply: We have included this accordingly.

4. Please abbreviate liters to L (L, mL, µL) to avoid confusion.

Autor reply: We have changed this accordingly.

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Autor reply: We have changed this accordingly.

6. 1.1, 3.2.1: Please write the text in the imperative tense. Any text that cannot be written in the imperative tense may be added as a "NOTE".

Autor reply: We have changed this accordingly.

7. 1.2.3: Please spell out IPTG.

Autor reply: We have changed this accordingly (line 135).

8. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.

Autor reply: We have changed this accordingly.

9. 1.2.4, 2.1.1.1: Please provide the composition of sonication/AIEX buffer A.

Autor reply: We included the preparations in the protocol (line 140ff, 165ff).

10. 2.1.1.2: What is the molecular weight cut-off of the dialysis tubing?

Autor reply: We included this information (line 172).

11. 2.1.1.5: Please provide the composition of dialysate buffer.

Autor reply: We have changed this accordingly (line 183).

12. 3.1.1: Please describe how this is actually done.

Autor reply: We have changed this accordingly.

13. 5.2.1: Please describe how to stimulate cells.

Autor reply: We have changed this accordingly.

14. 5.2.2: Please describe how to harvest supernatants.

Autor reply: We have changed this accordingly.

15. 2.1.1.6, 2.2.2.1: Should be "µm" instead of "µM".

Autor reply: We have changed this accordingly.

16. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.

Autor reply: We have changed this accordingly.

17. Please apply single line spacing throughout the manuscript, and include single-line spaces between all paragraphs, headings, steps, etc.

Autor reply: We have changed this accordingly.

18. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

19. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Notes cannot usually be filmed and should be excluded from the highlighting.

20. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

Autor reply: We have included this accordingly.

21. Figure 4 and Figure 5: Please abbreviate liters to L (L, mL, µL) to avoid confusion.

Autor reply: We have changed this accordingly.

22. Please include buffers and solutions information in a separate table in the form of an xlsx file and reference it in the manuscript.

Autor reply: We have included this accordingly.

23. Please remove the embedded figure(s) from the manuscript.

Autor reply: We have changed this accordingly.

24. Please remove the embedded table(s) from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text. Please reference them in the manuscript.

Autor reply: We have changed this accordingly.

25. Please include at least one paragraph of text to explain the Representative Results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures.

Autor reply: We have changed this accordingly.

26. Discussion: As we are a methods journal, please also discuss critical steps within the protocol, any modifications and troubleshooting of the technique, and any limitations of the technique.

Autor reply: We have changed this accordingly.

27. Please revise the Acknowledgements section to include any acknowledgments and all funding sources for this work.

Autor reply: We have included this accordingly.

28. References: Please do not abbreviate journal titles.

Autor reply: We have changed this accordingly.

Reviewer #1:

<u>Minor Concerns:</u>

1. The abstract is rather an introduction and not containing the scope of the paper.

Autor reply: We rewrote the abstract to meet this point.

2. Introduction: This part could benefit from an explanation of the DAMP terminus, involving the intracellular functions of S100A12 and how the extracellular milieu is shaping the oligomerization state in contrast to intracellular state.

Autor reply: We defined the 'DAMP' terminus in line 44 onwards. This was already included in the original manuscript.

3. figure 5: "untreated" in panels A and B are somehow misleading. To indicate LPS or S100A12 + treatment would be clearer.

Autor reply: We changed figure 5 accordingly.

4. Discussion: The authors do not discuss results of figure 5, which clearly shows effective endotoxin removal in the purification process and oligomerization dependent functional effects of S100A12, which should be stressed in the Discussion.

Autor reply: We now discussed results of figure 5 in line 383 onwards. However, as we have been requested to contribute a protocol paper we did not include further discussion on specific scientific findings as we do not want to recapitulate what is already published (Kessel et al., JACI, 2018).

Reviewer #2:

Major Concerns:

1. *Introduction. " …. we recently demonstrated human monocytes to respond to S100A12 stimulation in an exclusively TLR4-dependent manner". The authors cite Ref. 16 in support of this statement. However, RAGE, another S100A12 receptor, was not investigated in Ref. 16. Thus, the adverb "exclusively" should be deleted from that sentence. Alternatively, the authors perform studies like those shown in Fig. 5B,C in the presence of a RAGE neutralizing antibody (and a TLR4 neutralizing antibody for comparison).*

   Autor reply: Most of our current data indeed point towards a strong dependence of S100A12-signaling on TLR4/CD14 (Kessel et al., JACI, 2018; Armaroli et al, A&R, 2019). However, it is correct that ref 16 does not include a head-to-head comparison of anti-RAGE vs anti-TLR4 antibodies. Therefore, we removed the term 'exclusively' in line 72.

2. *Fig. 5B shows that in the presence of Poly B, S100A12 (60 microg/ml) causes monocytes to release ca. 3500 pg/ml TNF-alpha (that is quite similar to 200 pg/ml LPS), whereas at the same dose (1 microM = 60 microg/ml) hexameric S100A12 causes the release of ca. 1500 pg/ml TNF-alpha (Fig. 5C). One understands that unfractionated S100A12 is more potent that hexameric S100A12 at stimulating TNF-alpha release? In addition, Fig. 5C shows that tetrameric S100A12 only minimally stimulates monocytes and dimeric S100A12 is not effective at all.*

   Autor reply: The responsiveness of primary human monocytes to stimulation is substantially affected by donor-to-donor variations. Thus, absolute amounts of TNF-alpha from different cell-stimulation experiments are difficult to compare and do not warrant such conclusion. We added a note in line 309ff to draw attention to the different sensitivities/responsiveness of the monocytes.

3. *One wonders if monocytes can ever come into contact with such enormous concentrations of S100A12 and calcium as those used here.*

Autor reply: The (high) concentrations of calcium and zinc used were selected only for methodological reasons in course of protein purification. These concentrations have proved beneficial in method development. Further discussion on oligomerization of S100A12 with physiological concentrations of calcium und zinc (intra- and extracellular environment) have been published elsewhere (e.g. Kessel et al., JACI, 2018).
We comment on this in line 225, 452ff and 492ff.

Reviewer #3:

Minor Concerns:

1. *Could the authors justify the reason of using high concentration of calcium (25 mM calcium) in their experiments. Couldn't 2-3 mM calcium be sufficient?*

   Autor reply: Please refer to our reply to question #3 by reviewer #2.

2) In the SDS-PAGE gels presented in Figures 1 and 2, the authors should indicate what the numbers indicated on top of the gel represent: are those elution volumes or tube numbers?

Autor reply: We changed this accordingly.

3) In figure 5, it would be useful to indicate the different treatment conditions below the X-axis of the bar plots, because in certain conditions the bars are not visible.

Autor reply: We changed this accordingly.

Reviewer #4:

Minor Concerns to correct and to address:

1. *The manuscript describes a protocol that could be useful for other scientist. However, I would like to draw the attention of the authors to the fact that a protocol to isolate tag-free recombinant S100A12 (together with all other S100 proteins) produced in E. coli has recently been published and should be referenced (Kiss et al, Methods Mol Biol. 2019; 1929:325-338. doi: 10.1007/978-1-4939-9030-6_21).*

   Autor reply: Thank you, we included this in lines 104 onwards.

2. *The reviewer is curious to know why chemical xlinking was applied before purification of the oligomers? It is obvious that this step stabilizes the metal-induced transition of the different oligomeric forms. Are these form in a dynamic equilibrium that should prevent separation by gel filtration? The authors should comment on it.*

   Autor reply: S100A12 oligomers are certainly existing in a dynamic equilibrium depending on given ion concentrations. Thus, in case ion concentrations cannot be maintained throughout the purification procedure this precludes separation

of defined oligomers. In our protocol this is bypassed by chemical cross-linking prior to gel filtration.

3. *The extracellular physiological Ca- and Zn-ion concentrations are around 1 mM and in the micromolar range, respectively. Why is much higher Ca (and Zn) concentration needed to induce oligomerization?*

   Autor reply: That is correct. Please refer to our reply to question #3 by reviewer #2.

4. *Gel concentrations of SDS-PAGE runs in Fig 3A and 3B are different and not shown, neither the size of the markers on Fig 3B. Should be corrected.*

   Autor reply: Thank you, well spotted. We changed this accordingly.

5. *I assume that in the size exclusion chromatography of Fig 4A and 4B the same samples were run as analyzed in Fig. 3B (I surmised it from the Figure Legends). Why is then considerably less hexameric protein visible in the gel and more absorbance of this in the elution profile?*

   Autor reply: This is not the identical sample. We have changed the figure legend to describe this more clearly.

6. *In Fig. 4C, the band (lane 2) at ~35 kDa is not explained. Is it a trimer? Can it be explained based on the current model of the tetramer?*

   Autor reply: We have included a short comment on this in line 390ff. Transition states, like trimers, have also been described elsewhere (Augner et al, PLoS One,2014; Lutzow et al, BMC Vet Res, 2008).

7. *A brief explanation is needed why TNFα production is declined by adding poly B in case of S100A12. The label of x axis is should be the same in Fig 5. for S100A12 and S100A12 oligomer concentrations (currently they are given in µg/ml and nM, respectively).*

   Autor reply: We have included a comment on polymyxin B in the discussion (line 472ff) and we have separated fig 5C from 5A and B, to underline the different purposes. In Fig 5A and B, we wanted to verify functionality and purity (absence of endotoxin) of the produced protein. In 5C (fig 6) we wanted to compare the different oligomers on monocytes, which is easier with equal molar concentrations. In line 392ff and 394ff we have included this information in the manuscript.

8. *The authors applied EndoLISA endotoxin test to remove bacterial endotoxins/LPS. It is mentioned that ion exchange chromatography eliminate the vast majority of contamination, which is followed by an additional - hydrophobic interaction - chromatography step. The authors do not provide data about endotoxin concentration after the second purification step.*

   Autor reply: We have included data on endotoxin measurements during the process in line 379ff. However, this does not include a measurement following hydrophobic-interaction chromatography, as this step is not important for endotoxin removal.

Reviewer #5:

Major Concerns:

1. *There is another JOVE article that describes the expression and purification of S100A12 from E coli (https://www.jove.com/video/55557/expression-purification-and-antimicrobial-activity-of-s100a12) that is not cited in this manuscript. c*
   However, the previous method does not describe any endotoxin removal steps. This protocol should be cited and the authors should point out how their current method includes additional procedures.

   Autor reply: Thank you, we reference this article in the revised manuscript (line 104ff).

Minor Concerns:

1. *Line 48 (and subsequent text)- most S100 proteins are obligate dimers, hence "monomer" is not precise in its usage. The accepted term should be subunit or protomer.*

   Autor reply: As also done in in other publications (i.e. ref 13, Vogl et al, J Biol Chem) we term the 92 aa S100A12 polypeptide 'monomer'.

2. *Line 52 S100A12 binds Cu2+ but does not bind Mn2+, this is established in reference 6*

   Autor reply: Thank you, we changed this in line 60 onwards.

3. *Lines 54-57 This statement is incorrect, Ca2+ increases S100A12 affinity but is not a prerequisite for binding. Reference 6 describes fluorescent indicator competition assays that demonstrate Kd for Zn2+ is nM to sub nM even in absence of Ca2+*

   Autor reply: Thank you, we re-phrased this (line 64).

Protocol Section Numbers

1.2.1 - define vessel used for 5 mL growth and specify "vigorous shaking"

Autor reply: We changed this accordingly (line 130f).

3.2.2 Define what percent yield of protein is obtained from filtration step.

Autor reply: We changed this accordingly (line 256ff).

5.2 and figure 5. Details should be provided on the statistical analysis.

Autor reply: The depicted data are representative results from single experiments and thus do not warrant any statistical testing.

Figures

1. Figure 4 Title "Separation of S100A12 complex forms" is awkward. Suggest Separation of S100A12 oligomers

   Autor reply: We changed this accordingly.

2. Figure 5b specify S100A12 (oligomer or not) used in assay

   Autor reply: We changed this accordingly (line 443).

3. Figure 5c, x-axis is in nM whereas previous panels are ug/mL. This makes it difficult to compare. For example, the signal is much higher in panel B than for any oligomers in panel C.

   Autor reply: Please refer to our reply to question #2 by reviewer #2 and question #7 by reviewer #4.

(A) Stock solutions

Ampicillin stock solution (100 mg/mL)
Dissolve 1 g of ampicillin (sodium salt) in deionized water to make a final volume of 10 mL.
Filter sterilize (0.22 µm), and store aliquots at -20 °C.

1 M $CaCl_2$ dihydrate (MW: 147.02 g/mol)
Dissolve 14.7 g of calcium chloride dihydrate in 1000 mL of deionized water.

500 mM ethylenediamine tetraacetic acid disodium salt dihydrate (EDTA) (MW: 372.24 g/mol)
Dissolve 90.6 g of EDTA in 300 mL of deionized water
Adjust pH to 8.0 with NaOH (EDTA will go into solution as the pH nears 8.0)
Fill up to a final volume of 500 mL with deionized water

1 M isopropyl-ß-D-thiogalactopyranosid (IPTG)
Dissolve 2.38 g of IPTG in 10 mL of deionized water.
Filter sterilize (0.22 µm), and store aliquots at -20 °C.

1 M NaCl (MW: 58.44 g/mol)
Dissolve 5.8 g of sodium chloride in 80 mL of deionized water
Fill up to a final volume of 100 mL with deionized water

1 M Tris buffer (MW: 121.14 g/mol)
Dissolve 12.1 g of Tris base in 80 mL of deionized water
Adjust pH with HCl
Fill up to a final volume of 100 mL with deionized water

100 mM $ZnCl_2$ (MW: 136.28 g/mol)
Dissolve 1.36 g of zinc chloride in 100 mL of deionized water.

(B) Buffers

AIEX buffer A
20 mM Tris
1 mM EDTA
1 mM EGTA
Dissolve in deionized water (2/3 of final volume)
Adjust pH to 8.5 with HCl
Fill up to final volume with deionized water
Filter through 0.45 µm and degas

AIEX buffer B
20 mM Tris

1 mM EDTA
1 mM EGTA
1 M NaCl
Dissolve in deionized water (2/3 of final volume)
Adjust pH to 8.5 with HCl
Fill up to final volume with deionized water
Filter through 0.45 µm and degas

HIC buffer A

20 mM Tris
140 mM NaCl
25 mM $CaCl_2$
· dissolve in deionized water (2/3 of final volume)
· adjust pH to 7.5 with HCl
· fill up to final volume with deionized water
· filter through 0.45 µm and degas

HIC buffer B

20 mM Tris
140 mM NaCl
50 mM EDTA
Dissolve in deionized water (2/3 of final volume)
Adjust pH to 7.0 with HCl
Fill up to final volume with deionized water
Filter through 0.45 µm and degas

HBS

20 mM Hepes
140 mM NaCl
Dissolve in deionized water (2/3 of final volume)
Adjust pH to 7.2 with NaOH
Fill up to final volume with deionized water
Filter through 0.45 µm and degas