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Selective enrichment of monospecific polyclonal antibodies for antibody-based proteomics efforts

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Abstract

A high stringency protocol, suitable for systematic purification of polyclonal antibodies, is described. The procedure is designed to allow the generation of target protein-specific antibodies suitable for functional annotation of proteins. Antibodies were generated by immunization with recombinantly produced affinity-tagged target proteins. To obtain stringent recovery of the antibodies, a two-step affinity chromatography principle was devised to first deplete the affinity tag-specific antibodies followed by a second step for affinity capture of the target protein-specific antibodies. An analytical dot–blot array system was developed to analyze the cross-reactivity of the affinity-purified antibodies. The results suggest that the protocol can be used in a highly parallel and automated manner to generate mono-specific polyclonal antibodies for large-scale, antibody-based proteomics efforts, i.e. affinity proteomics.

Keywords: Proteomics; Immobilized metal-ion affinity chromatography; Affinity chromatography; Antibodies; Protein epitope signature tags

1. Introduction

For decades antibodies have been invaluable tools for studying proteins because of the highly specific binding and strong affinity to their targets. Specific antibodies are being used in many different areas, such as in localization studies, isolation of native protein complexes and in purification procedures. Therapeutically, they are used in a number of applications including passive immunization in case of bacterial or viral infections, targeting to certain cells (e.g. tumor cells), selective delivery of diagnostic agents or in specific manipulation of a target antigen. Often, difficulties in purifying antibodies have hindered their use in therapeutic applications, which emphasizes the need of effective and reliable purification strategies. When little or nothing is known about a certain protein, localization information will enhance the understanding of the function of the protein significantly [1]. By using monospecific polyclonal antibodies, localization data can be achieved not only on a tissue or cell, but also on a subcellular level [2,3]. With the human DNA sequence at hand we are now challenging the mapping of the human proteome including thousands of proteins with totally unknown localization and function.

Recently, a general tool for genome-based proteomics has been proposed [3], in which protein-specific affinity reagents are generated, which subsequently can be used in a step-wise manner for a wide range of functional and biochemical studies. With a combination of bioinformatics tools and streamlined procedures for cDNA cloning and protein expression, antibodies specific to a large portion of the encoded proteins of human chromosome 21 were generated in an affinity proteomics approach. The concept was based on the generation of polyclonal antibodies towards 100–150 amino acid fragments, called protein epitope signature tags (PrESTs), selected based on their relative low homology to other proteins in the human proteome. The PrESTs were fused to an albumin-binding protein (ABP) for obtaining an increased immune response [4]. In addition, a His_6-tag was included to make it possible to purify the fusion protein under denaturing conditions, since a high proportion of the fusion proteins form inclusion bodies when produced in *Escherichia coli*.

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The use of polyclonal antibodies for such functional proteomics efforts has some advantages when compared to monoclonal antibodies. Cumbersome screening methods to select the most optimal reagent are avoided. Additionally, polyclonal antibodies are more likely to recognize its target in a wide range of functional assays resulting in both native and partly denatured proteins. In most cases, multi-epitope antibodies are also more reliable for protein "pull-out" experiments, in which the antibodies can be used to affinity purify a specific protein from a complex biosample, followed by various biochemical analyses, such as analysis of post-translational modifications using mass spectrometry [5]. However, a disadvantage with a polyclonal antibody strategy is that the generated affinity reagents obtained after immunization schemes often give cross-reactivity to other proteins or show high background binding. It is noteworthy that less than 5% of polyclonal antibodies in a typical antiserum are directed towards a specific target [6]. Thus, the use of raw antiserum would often lead to cross-reactivity to unrelated targets in the biosamples. Obviously, this limits the usefulness of polyclonal antibodies for proteomics applications and leads to issues regarding quality assurance of the generated affinity reagents. Hence, there is a great need for affinity procedures in which the antiserum is purified using its interaction with the target protein in order to allow purification of the highly specific antibodies.

Several strategies have been investigated for affinity purification of polyclonal antibodies. Larsson et al. [2] described a system for generation of polyclonal antisera and affinity enrichment of the specific antibodies directed against gene products from a cDNA library. The antibodies generated to the fusion protein were purified using an immunoblot procedure with gel-purified antigen. Agaton et al. [3] used a similar strategy to obtain mono-specific polyclonal antibodies using a single expression vector. Unfortunately, the gel-based method is not suitable for high-throughput procedures and the antibodies directed against the fused affinity tags are not depleted in the procedure. An alternative strategy has therefore been tried involving dual expression concepts [7–9]. In this approach, the cDNA is expressed with two different affinity tags. One of the cDNA fusion proteins is used for the immunization and the other for the affinity purification. Although this results in a more specific antibody fraction, the concept leads to twice as many DNA constructs and protein expression and purification experiments. The dual expression concepts are therefore not optimal for large-scale projects in which cloning and expression steps should be kept to a minimum.

In this paper, we show that it is possible to separate antibodies of different specificity to the same immunogen into different fractions. This suggests that it is not necessary to use a second genetic design of the antigen in the purification step. The improved purification strategy shown in this study involves first a depletion step and second a capture step. In the depletion step, the antibodies with specificity towards the fusion partner (His_6 and ABP) are captured. In addition, serum albumin, which is the most abundant protein in the sera, is trapped. The specific antibodies are subsequently affinity-purified in a second step using the intact fusion protein.

We have investigated different approaches of purification and optimized the system using two protein fragments (PrESTs) representing the human proteins SOD1 and CCT8. SOD1 and CCT8 are well-characterized proteins of known functions and were therefore chosen to evaluate our concept.

We show that the antibodies captured in the depletion and capture step respectively are reactive to different parts of the fusion proteins used as immunogens. We also show by utilizing a dot–blot array containing multiple antigens (PrESTs) that the monospecific polyclonal antibodies achieved indeed recognize their specific antigen only and suggest that the strategy developed would be suitable for automation in large-scale applications.

2. Experimental

2.1. Expression, purification and immunization of fusion proteins

Plasmids encoding the PrESTs representing the proteins SOD1 and CCT8, earlier constructed [3] from the original expression vector pAff8c [2] and pAff10c [8], were transformed to the *E. coli* strain BL21(DE3) (Novagen, Madison, WI, USA). For protein production, cells were grown overnight in 30 g/l tryptic soy broth (TSB) (Difco, Detroit, MI, USA) containing 50 μg/ml kanamycin and 34 μg/ml chloramphenicol at 37 °C. From each clone, 5 ml overnight culture was used to inoculate 500 ml TSB + yeast extract supplemented with antibiotics as above. Cultures were grown at 37 °C to an optical density (OD) between 0.8 and 1.0. At this OD, protein expression was induced by addition of IPTG (Apollo Scientific, Whaley Bridge, UK) to a final concentration of 1 mM and the cultures were incubated for another 4 h before harvested by centrifugation at 2000 × *g* for 10 min. Cell pellets were resuspended in 40 ml lysis buffer (50 mM NaH_2PO_4, 10 mM Tris–HCl, 100 mM NaCl, 6 M guanidinium–HCl, pH 8.0). Cells in lysis buffer were sonicated (High Intensity Ultrasonic Processors, 750 W model, Sonics & Materials, Newtown, CT, USA) prior addition of 10 mM β-mercapthoethanol and the suspensions were incubated on a stirrer for 2 h at room temperature. Protein solutions were clarified by centrifugation at 12,000 × *g* for 10 min and the supernatants filtered on 0.45 μm pore size filter (Satorius, 37 070 Goettingen, Germany). To purify the His_6-tagged proteins, 2.5 ml columns with TALON (Clontech Lab., Palo Alto, CA, USA) for immobilized metal-ion affinity chromatography (IMAC) were used. The IMAC procedure was performed as recommended by the manufacturer. The protein contents of all eluted protein fractions were analyzed by absorbance measurements at 280 nm. Protein containing fractions were

pooled and diluted with phosphate-buffered saline (PBS) (1.9 mM NaH_2PO_4, 8.1 mM Na_2HPO_4, 154 mM NaCl, pH 7.25) to an urea concentration of 2 M and stored at −20 °C. The dual affinity handle, His_6-ABP, in pAff8c, was also expressed and purified according to the same protocol.

Additionally, IMAC-purified His_6-ABP-SOD1:PrEST from a 500 ml culture was affinity-purified on a column packed with human serum albumin (HSA) Sepharose. Before applied on the HSA column, the IMAC-purified protein, in diluted elution buffer, was dialyzed against 3× 800 ml TST (50 mM Tris–HCl, pH 7.5, 2 M NaCl, 0.05% Tween 20) under stirring at +4 °C for 2× 60 min plus over night. The His_6-ABP-fused SOD1:PrEST was loaded on a 2.5 ml HSA Sepharose column pre-equilibrated with TST. Unbound materials were washed away with 25 ml TST before elution in 1 ml fractions with 0.5 M HAc, pH 2.5.

Purified proteins were analyzed in terms of size, purity and relative concentrations by separation on 20% homogenous sodium dodecyl sulfate–polyacrylamide gel electrophonesis (SDS–PAGE) gels and Coomassie staining in the Phast system according to the instructions of the manufacturer (Amersham Biotechnology, Uppsala, Sweden).

New Zealand rabbits, two per antigen, were immunized with the IMAC-purified PrEST fusions by AgriSera (Vännäs, Sweden) as previously described [2].

2.2. Preparation of affinity matrices

Specific affinity purification matrices were obtained using prepacked *N*-hydroxysuccinimide (NHS)-activated columns (1 ml HiTrap HP, Amersham). All proteins to be coupled were concentrated on vivapore concentrators 10–20 ml, 7.5 kDa cut-off (MWCO) (Vivascience, Hannover, Germany) to at least 1 mg/ml before buffer exchange to coupling buffer (0.2 M $NaHCO_3$, 0.5 M NaCl, 2 M urea, pH 8,3) using PD-10 desalting columns (Amersham). Coupling of the proteins was performed according to manufacturer's guidelines. Briefly, the columns were washed with 6 ml 1 mM HCl prior injection of 1 ml protein solution and then incubated for 30 min at room temperature. Excess of activated groups were deactivated by ethanolamine and uncoupled ligands were washed out by pulsing three times with buffer A (0.5 M ethanolamine, 0.5 M NaCl, pH 8.3) and buffer B (0.1 M HAc, 0.5 M NaCl, pH 4) including an extended incubation (30 min) with buffer A after the first pulse. The coupled columns were stored in PBS supplemented with 20% ethanol at +4 °C.

2.3. Purification of antisera

Unpurified antisera from animals immunized with the same antigen were tested on a panel of protein samples from homogenized human tissues in Western blots and antisera giving most specific or most intensive staining (data not shown) were selected for further purification.

The columns were pulsed with three column volumes (CVs) (6 ml in total) antibody elution buffer (Ab-elution buffer) (0.2 M glycine, 1 mM EGTA, pH 2.5) and PBS and then equilibrated with 10 CV PBS. Antisera were diluted, 2 ml sera + 8 ml PBS, and applied to the affinity columns at a flow rate of 10 ml/h using a peristaltic pump P-1 (Pharmacia Fine Chemicals, Sweden). Unbound materials were washed away with 25 CVs PBS containing 0.05% Tween 20 (PBST) and 5 CVs PBS at a flow rate of 1 ml/min. The capture column was separated from the depletion column in order to elute bound materials separately. Elution was done with 250 μl fractions of Ab-elution buffer into tubes containing 12.5 μl 1 M Tris–HCl and 25 μl 10× PBS to adjust pH to approximately 8.0. Antibody-containing fractions were detected by absorption measurements at 280 nm. NaN_3, 0.02%, was added, to the purified antibodies before refrigeration.

2.3.1. Strategy 1a, 1b and 2

Antisera were purified on single NHS-activated 1 ml HiTrap columns (Amersham) coupled with the PrESTs corresponding to CCT8 or SOD1 fused to His_6-ABP (strategy 1a and 1b) or to His_6 (strategy 2) respectively (Fig. 1).

2.3.2. Strategy 3

Depletion and capture purifications of antisera were performed on two columns in a serial mode. The first column was coupled with the dual affinity tag, His_6-ABP, and on the second column, the specific His_6-ABP-PrEST fusion protein was coupled (Fig. 1).

2.4. Western blot analysis

2.4.1. Tissue Western

Tissue homogenisates representing the human tissues colon, kidney and liver were separated under denaturing conditions by electrophoresis on NuPage 3–8% Tris–acetate gels (Novex, Invitrogen, Carlsbad, CA, USA) and electro-blotted to Immobilon-P membranes (Millipore, Bedford, MA, USA). The membranes were blocked in PBS containing 5% milk proteins (Semper, Sweden) for 60 min at room temperature before incubated for 60 min on a shaker at room temperature with the purified antibodies or, as a control, with the unpurified antisera. The membranes were washed in PBST before incubation for 45 min at room temperature with the secondary antibody (anti-rabbit IgG conjugated to horseradish peroxidase 1:3000) (DAKO A-S, Glostrup, Denmark). Excess of secondary antibody was washed away with PBST. SuperSignal West Dura (Pierce, Rockford, IL, USA) was used as detection system and digital images of the chemiluminescense were monitored using a ChemiImager (Alpha Innotech, San Leandro, CA, USA).

2.4.2. Validation blots of purified antibodies

To investigate the purity of the antibodies, 1 μg of each PrEST fusion protein were run on 10–20% Tris–Glycine

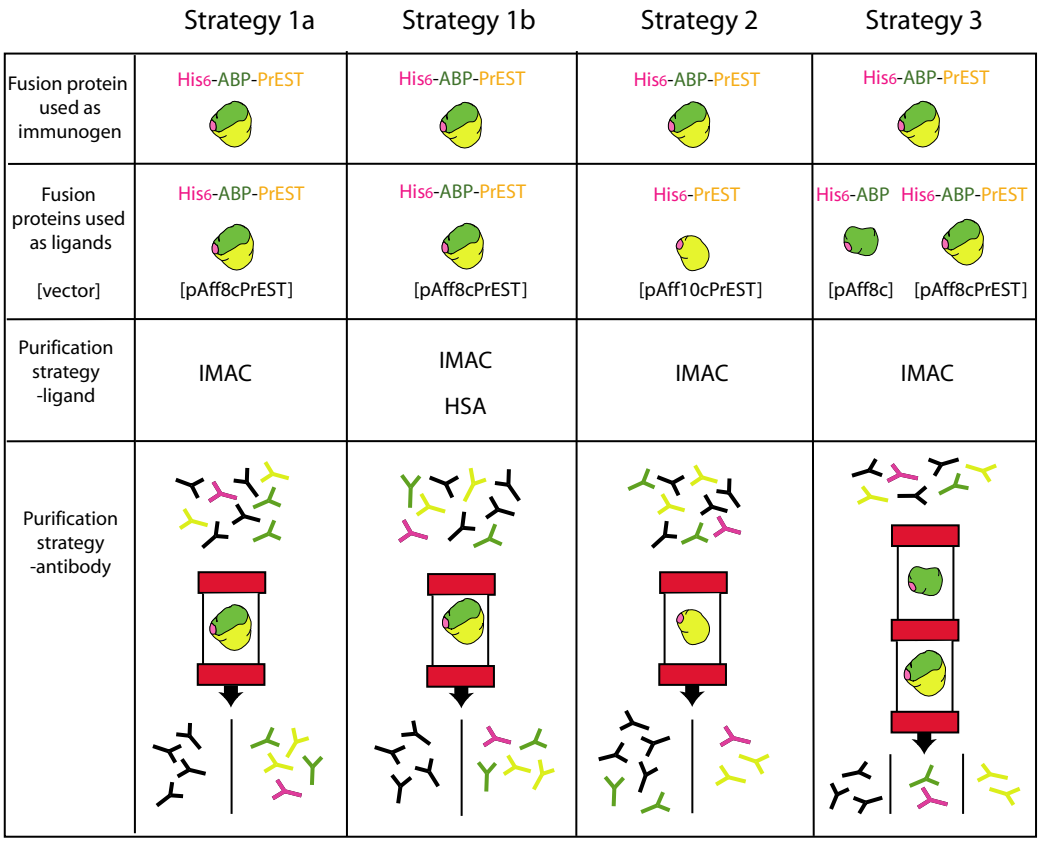

Fig. 1. Overview of the purification strategies investigated. The different fusion proteins consist of His_6 (pink) and/or ABP (green) and/or PrEST (yellow). The purified antibodies are color coded according to specificity. In strategy 1, the serum is affinity-purified using the immobilized antigen as ligand, His_6-ABP-PrEST, which is purified once (1a); alternatively the antigen is purified twice (1b). In strategy 2, the serum is purified using the His_6-PrEST as ligand (purified once). In strategy 3, the serum is first purified using immobilized His_6-ABP for depletion of albumin and of antibodies directed against the common part of the PrEST fusion proteins, and subsequently, the serum is purified against immobilized His_6-ABP-PrEST for capture of the antibodies specific to the PrEST. Both His_6-ABP and the antigen, His_6-ABP-PrEST, are purified once before coupling to the matrix.

gels (Novex), electroblotted onto nitrocellulose membranes (Novex) and incubated with their respective antisera. The dot–blot array membranes were treated similarly, but instead of running the antigens on SDS–PAGE, 0.5–1 μg of each fusion protein/full-length protein was dotted onto nitrocellulose membranes. Thereafter, the membranes were blocked with 5% milk proteins (Semper) in PBST for 60 min. Excess milk proteins were washed away by PBST and the membranes were incubated with their respective unpurified antisera or purified antibodies for 60 min at room temperature. In order to make the Western blots with raw sera and purified antibodies comparable, the achieved amount of monospecific antibodies after purification was assumed to be 100%. Consequently, the raw sera and purified antibody fractions were normalized to account for volume changes in the purification procedure. The membranes were washed with PBST, incubated with the secondary antibody (goat anti-rabbit IgG-alkaline phosphatase; 1:10,000; Sigma) for 60 min at room temperature, washed again and finally developed with 5-bromo-4-chloro-3-indolyl phosphate–nitro blue tetrazolium (BCIP–NBT) tablets (Sigma) according to the supplier's recommendations.

3. Results

The quality of polyclonal antibody preparations has proven to depend on the degree of stringency in the antibody purifications. In an effort to develop a general strategy for the generation of high quality antibodies we have investigated three different antibody purification strategies of polyclonal sera (Fig. 1). Strategy 1a: affinity purification of antibodies using a column coupled with the immunogen (His_6-ABP-PrEST) as affinity ligand, which has been purified once prior to coupling. Strategy 1b: the same strategy as 1a, with the exception that the affinity ligand has been purified twice. Strategy 2: a fusion protein excluding ABP is used as affinity ligand (His_6-PrEST). Strategy 3: two affinity columns are used in a dual purification approach. The first column is coupled with a fusion protein excluding the PrEST (His_6-ABP) and the second with the same affinity ligand as in strategy 1a and b, i.e. the fusion protein used for immunization (His_6-ABP-PrEST).

Two vector systems were used; pAff8c [2] and pAff10c [8] (Fig. 1). In pAff8c, the PrEST is cloned in frame with the dual affinity tag containing His_6, ABP and an additional

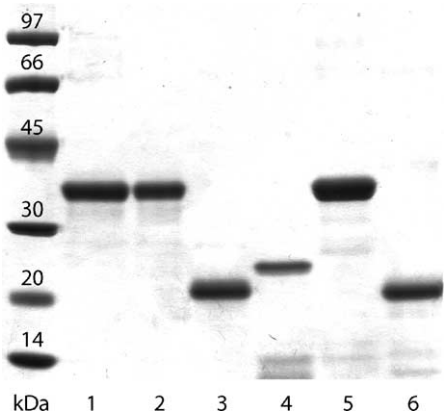

Fig. 2. An SDS–PAGE stained with Coomassie blue showing IMAC-purified fusion proteins. Lane 1: His_6-ABP-SOD1:PrEST, lane 2: His_6-ABP-SOD1:PrEST also purified on HSA sepharose, lane 3: His_6-SOD1:PrEST, lane 4: His_6-ABP, lane 5: His_6-ABP-CCT8:PrEST; and lane 6: His_6-CCT8:PrEST.

linker region. In pAff10c, ABP is excluded. Expression using pAff8c (no insert) results in the dual tag protein, which was used for the depletion procedure. The different fusion proteins were coupled on NHS-activated columns and after loading of unpurified sera and extensive washing of the columns, the captured antibodies were eluted at pH 2.5.

Polyclonal antibodies raised against PrESTs from two known genes located on chromosome 21 (SOD1 and CCT8) were chosen for the optimization. The PrEST fusions used as affinity ligands were purified in a single step procedure, taking advantage of the His_6-tag (strategy 1a, 2, 3). During the IMAC purification there is a probability of co-purifying and thereby co-immunizing *E. coli* proteins, which can cause background signals when using the antibodies in, for example, immunohistochemical studies. Since the SOD1:PrEST fusion protein is a soluble protein fragment and could be subjected to HSA-purification using the ABP-tag, the necessity of a more stringently-purified PrEST for antibody capture was tested. Hence, His_6-ABP-SOD1:PrEST was

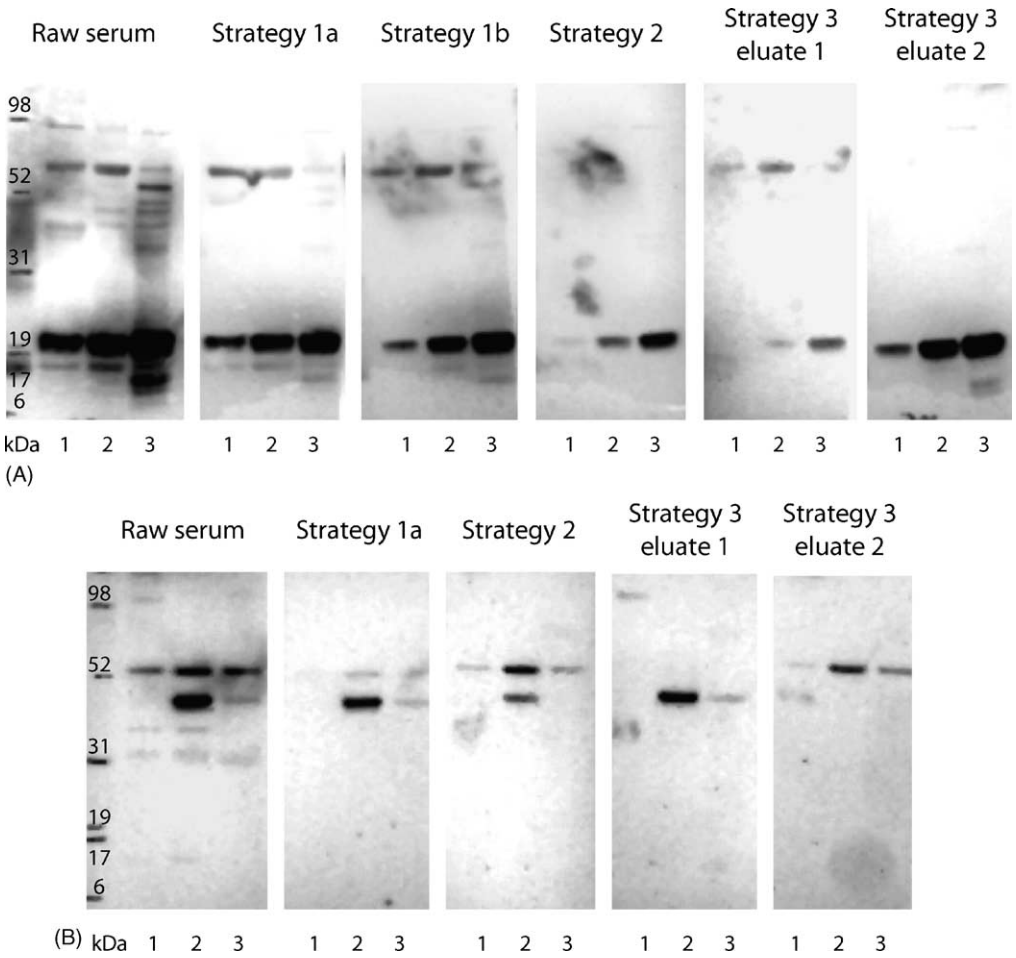

Fig. 3. Tissue Western comparing specificity of raw serum and purified antibodies. Protein lysates from the following tissues were loaded as follows: lane 1, colon; lane 2, kidney; lane 3, liver. (A) Detection of SOD1 using unpurified α-SOD1-antibodies (panel I), purified by strategy 1a (panel II), by strategy 1b (panel III), by strategy 2 (panel IV) and by strategy 3 (panel VI). Panel V shows activity of the eluate from the depletion column in strategy 3. (B) Detection of CCT8 using unpurified α-CCT8-antibodies (panel I), purified by strategy 1a (panel II), by strategy 2 (panel III) and by strategy 3 (panel V). Panel IV shows activity of the eluate from the depletion column in strategy 3.

purified by IMAC using the His_6-tag and subsequently by HSA-purification, taking advantage of the ABP-tag (strategy 1b). The SOD1:PrEST fusion protein was then coupled to the column matrix using NHS-chemistry. Fig. 2 shows the different fusion proteins from both pAff8c and pAff10c purified by IMAC and also the double-purified SOD1:PrEST fusion protein run on an SDS–PAGE gel. In order to analyze the specificity of the purified polyclonal antibodies, a tissue Western was performed on human protein lysates from three different tissues, colon, kidney and liver (Fig. 3).

Fig. 3A shows the specificity of the unpurified SOD1 antiserum and of the antibodies from the different purification strategies. No improvement by the second purification of the SOD1 antigen could be detected. A band corresponding to SOD1 (super oxide dismutase 1, $M_r \approx 16{,}000$) was observed in all three tissues. Another band can also be visualized at approximately M_r 60,000 for the polyclonal antibodies purified according to the first strategy (Fig. 3A, panels II and III), i.e. when the antibodies are purified against the fusion protein used for immunization (Fig. 1, first strategy). The 60 kDa band cannot be seen when the polyclonal antibodies have been purified against the PrEST fused to the His_6-tag only (Fig. 3A, panel IV; Fig. 1, second strategy) or when they have been subjected to the depletion procedure before entering the column coupled with the His_6-ABP-PrEST (Fig. 3A, panel VI; Fig. 1, third strategy). It is obvious that the antibodies directed against an M_r 60,000 protein are eluted from the column coupled with His_6-ABP (Fig. 3A, panel V) and thus are most likely directed against the ABP-tag and not to a protein homologous to a co-immunized *E. coli* protein or the His_6-tag. Almost the same pattern is shown for the other antiserum chosen for this study, directed against CCT8 (Fig. 3B). CCT8 is an M_r 59,000 molecular chaperone also detected in colon, kidney and liver, according to the figure, although faint in colon. In this blot there is a band appearing an M_r 45,000, which does not disappear when the antibodies are purified according to the second strategy (panel III), but to the third (panel V). The false positive antibodies directed against a 45 kDa human protein do clearly elute from the His_6-ABP-column (panel IV), showing either His_6-tag reactivity or specificity towards the linker region originating from the expression vector.

We concluded to continue with the third purification strategy for further investigation of the quality of the purified antibodies. The antigens from Fig. 2 were separated on an SDS–PAGE gel, blotted onto nitrocellulose membranes and incubated with unpurified and purified antibodies (Fig. 4). For both antigens, it is obvious that the target-specific antibodies are enriched after purification (Fig. 4A, lane 3; Fig. 4B, lane 2) and the antibodies directed against the fusion partner completely depleted (Fig. 4A, lane 4; Fig. 4B, lane 3).

As an ultimate quality control of the purified polyclonal antibodies, dot–blot arrays were constructed visualizing the specificity of the antibodies against the target (Fig. 5). Different His_6-ABP-PrEST proteins and commercially available full-length proteins were manually spotted in duplicates onto nitrocellulose membranes and the membranes were incubated with antibodies either only purified against their immunogen, according to strategy 1, or in combination with the depletion step removing tag-specific antibodies according to the third strategy (Fig. 1). Fig. 5A shows the specificity of α-SOD1 polyclonal antibodies purified according to strategy 1. The reactivity of the antibodies purified from the same serum, but according to strategy 3, is shown in Fig. 5B. Fig. 5C and 5D show the specificity of α-CCT8 polyclonal antibodies purified according to strategies 1 and 3, respectively. As seen in Fig. 5B and D, the purified antibodies only recognized the constructs including the target protein. Notable is that α-SOD1 also recognized the native SOD1 protein. No background staining could be seen towards the other PrEST-fusions containing the dual

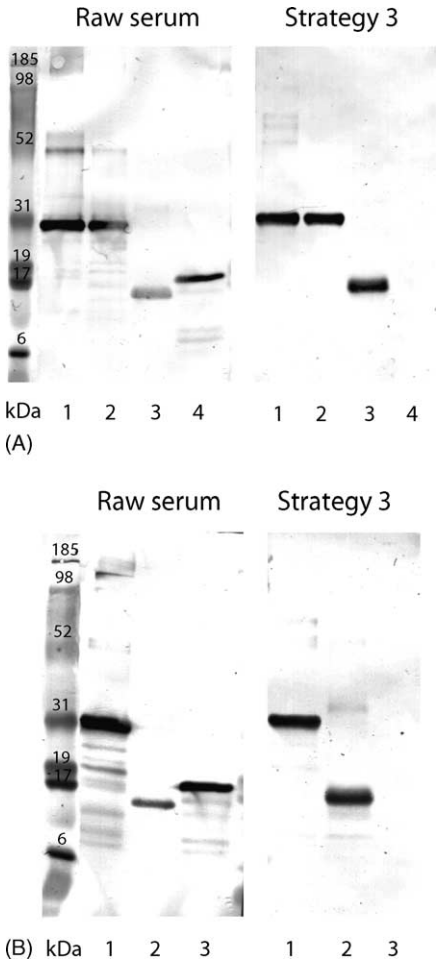

Fig. 4. Western blot showing enrichment of target-specific antibodies and depletion of the tag specific. (A) α-SOD1-antibodies recognizing their targets when unpurified (panel I) and purified according to strategy 3 (panel II). Targets loaded in lane 1: His_6-ABP-SOD1:PrEST, lane 2: double-purified His_6-ABP-SOD1:PrEST, lane 3: His_6-SOD1:PrEST and in lane 4: His_6-ABP. (B) α-CCT8-antibodies recognizing their targets when unpurified (panel I) and purified (panel II). Targets loaded in lane 1: His_6-ABP-CCT8:PrEST, lane 2: His_6-CCT8:PrEST and in lane 3: His_6-ABP.

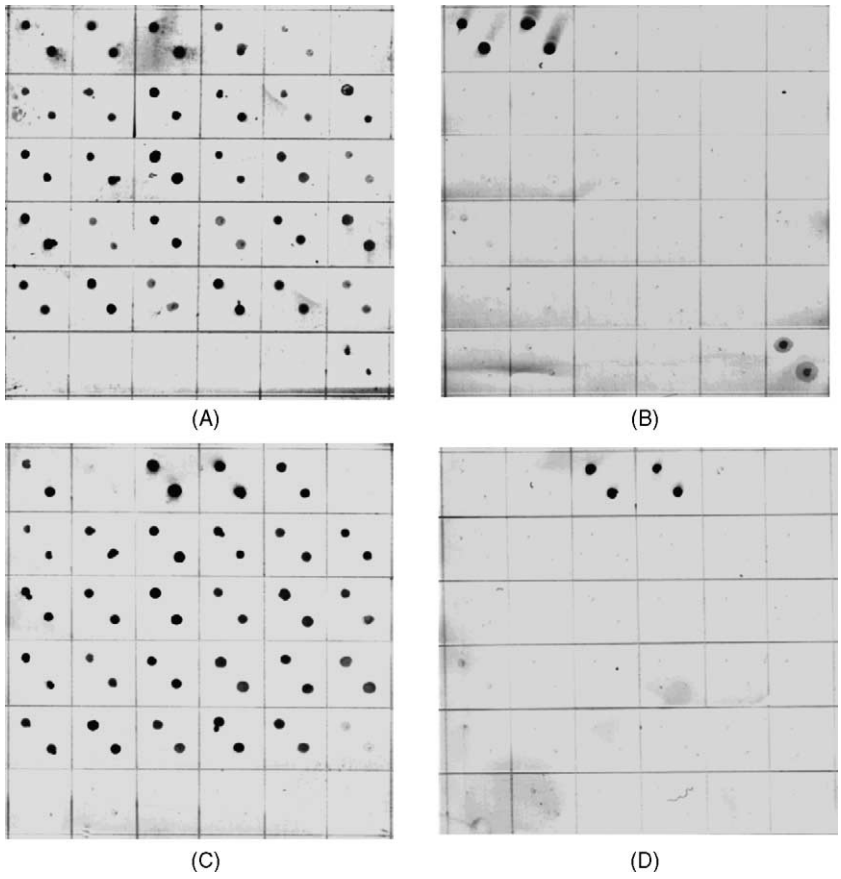

Fig. 5. Dot–blot protein array showing target specificity of antibodies. (A–D) PrESTs, tag fusions and full-length proteins immobilized onto four nitrocellulose membranes in duplicates in the following order; row 1: His_6-ABP-SOD1:PrEST, His_6-SOD1:PrEST, His_6-ABP-CCT8:PrEST, His_6-CCT8:PrEST, His_6-ABP, bovine serum albumin; rows 2–5: His_6-ABP:PrESTs from 24 unrelated genes; row 6: ribonuclease A, aldolase, ovalbumin, thyroglobulin, chymotrypsinogen A, SOD1. Detection by α-SOD1 antibodies purified (A) according to strategy 1 (B) according to strategy 3. Detection by α-CCT8 antibodies purified (C) according to strategy 1 and (D) according to strategy 3.

affinity tag, which were strongly recognized by the one-step immunogen-affinity-purified antibodies (Fig. 5A and C).

4. Discussion

Here, we have developed a streamlined, high stringency purification strategy for polyclonal antibodies, aimed to purify affinity reagents in a high-throughput manner. We have improved the purification procedures of polyclonal antisera to enrich the proportion of target (PrEST)-specific antibodies and thus reduce the cross-reactivity of the final antibody preparation. The PrESTs are expressed in fusion to a dual affinity tag consisting of ABP which has been shown to have an immunostimulating effect [4] and a His_6-tag, allowing IMAC purification. The presence of the His_6-tag is utterly convenient when working under denaturing conditions [10] which is the reason for keeping the tag as a part of the immunogen. Immunization with these proteins results in antibodies raised against the whole fusion protein. Thus, there is a need for depletion of antibodies directed against epitopes not originating from the target protein.

We have shown that the depletion of antibodies directed against the His_6-tag as well as ABP is necessary to avoid cross-reactivity to other proteins and background binding when using the antibodies for detection (Fig. 3). The additional band of approximately M_r 60,000 appearing in the tissue Western using α-SOD1 antibodies most probably represents ABP-specificity (Fig. 3A). When analyzing CCT8-specificity (Fig. 3B), it was concluded that antibodies most likely directed towards the His_6-tag possess cross-reactivity with an unexpected human protein of about M_r 48,000. These antibodies could easily be depleted as a result of the purification according to strategy 3 (panels III–V). Furthermore, we have shown that co-purified and thereby co-immunizied *E. coli* proteins from the single IMAC purification of the SOD1 antigen do not cause any background signals when using this high stringency antibody purification protocol.

Chromatography columns have proven to be a convenient way of coupling the affinity ligands in comparison to immobilization on nitrocellulose membranes as previously described by Larsson et al. [2]. Immobilizing PrESTs as affinity ligands on chromatography columns allows for

the use of larger amounts of antiserum, since scaling up the columns is relatively easy. This is particularly important when the titers of monospecific antibodies are low. Most steps in the protocols used in the presented paper are adaptable for automation and thereby also suitable for high-throughput applications, still yielding very specific affinity reagents.

A possible extension of this purification strategy would be to immobilize the purified antibodies on a matrix and use them for purification of full-length proteins from various tissues. As shown in Fig. 5, the monospecific antibodies directed against the PrEST from SOD1 recognized the native commercial protein (no commercial protein could be found for CCT8). Thus, by using these antibodies in a "pull-out" strategy, the native protein can be studied with relation to post-translational modifications using mass spectrometry and other physiochemical methods [11]. If the affinity capture is performed during native conditions it is also possible to analyze protein complexes and thus map protein–protein intractions.

We have shown that our strategy of purifying polyclonal antibodies generates affinity reagents highly specific to the antigen of interest with no cross-reactivity to the antigen fusion partners, to PrESTs from other genes or to other native proteins chosen for the limited array used in the study. Obviously, it would be more adequate in the future with a larger array including many additional PrESTs and proteins. We believe that this strategy can be scaled up to purify polyclonal antisera on a whole proteome-scale.

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