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Self-Immobilizing Recombinant Antibody Fragments for Immunoaffinity Chromatography: Generic, Parallel, and Scalable Protein Purification

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We present the directed immobilization of recombinant antibody fragments as ligands for general immunoaffinity chromatography methods. It is based on fusion proteins of scFv fragments with several chitin-binding domains which can be immobilized directly from a crude bacterial lysate on inexpensive chitin beads for the purification of proteins without any gradient or detector. It has been used with a positive pressure manifold, allowing the parallel processing of 24 different samples on a milligram scale, as convenient as plasmid isolation. The method is demonstrated with several anti-protein antibodies. In addition, methods are presented of using an anti-His tag antibody either alone or directly coupled to IMAC to obtain very pure protein. As those methods are scalable, they should prove very useful in the parallel purification of natural and recombinant proteins on small scales (for proteomics), medium scales (for crystallography and NMR), and very large scales (for therapeutic proteins).

Key Words: directed protein immobilization; recombinant antibody fragment; scFv; immunoaffinity chromatography; fusion protein; chitin-binding domain.

Because of its high affinity and selectivity, immunoaffinity chromatography (IAC)[3] (1–4) could be in principle of great value in the rapid purification of proteins to high degrees of purity. The antibody could either be used as affinity matrix in a one-step purification of natural proteins or be used for recombinant proteins in conjunction with methods based on peptide tags (5, 6) or even be directed against the peptide tag itself, thereby using the same tag twice in an orthogonal manner, as each column will remove other impurities. However, because of the large investment of time and effort and thus cost in generating monoclonal antibodies (mAb) and producing them at the scale required, IAC has only rarely been used in the purification of proteins on milligram scales. As the mAb will normally be covalently coupled to expensive chemically activated column material the use of a fresh column for each experiment, even though desirable, would be prohibitive in terms of labor and cost.

Recently, progress in display technologies such as phage display (7, 8), ribosome display (9, 10), the protein fragment complementation assay (11), and the availability of antibody libraries (12, 13) as a source for virtually all antibodies has solved the problem of obtaining recombinant antibodies with the desired specificity. Therefore, IAC with antibody fragments would be particularly attractive for the parallel purification of proteins for proteomics projects. However, no convenient method has been described for immobilizing the recombinant antibody fragments. Random crosslinking (14–17) to the columns may obstruct the binding site, and the smaller the fragment, the larger the chance that the most reactive group may be positioned by

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[3] Abbreviations used: IAC, immunoaffinity chromatography; mAb, monoclonal antibodies; scFv, single-chain Fv; CBD, chitin-binding domain; IMAC, immobilized metal ion affinity chromatography; RBS, ribosome-binding site; IPTG, isopropyl β-D-thiogalactopyranoside; GFP, green fluorescent protein; cv, column vol; NTA, nitrilotriacetic acid; HuCAL, human combinatorial antibody library.

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chance such that it interferes with antigen binding. The directed immobilization strategies of mAbs (18) rely on their Fc parts, e.g., coupling via their carbohydrate moieties or coupling by first binding to protein A or protein G columns, followed by crosslinking. Evidently, these methods cannot be applied to recombinant antibody fragments. If instead a tag is used for directed immobilization of the antibody fragment, which would then be bound by another protein, e.g., streptavidin (19, 20), this other protein will have to be purified and immobilized first, dramatically adding to the cost of using such columns on a large scale.

In this paper we introduce a new concept to simultaneously solve all of the above problems and provide a general approach for quickly preparing any immunoaffinity column from recombinant antibody fragments expressed in *Escherichia coli* in a one-step procedure with inexpensive column material. Therefore, many different and even very large affinity columns can be prepared very fast, and a general technology for high-throughput parallel protein purification on multimilligram scales is obtained, as is needed, e.g., in structural genomics projects.

The concept relies on making fusion proteins of an antibody fragment with engineered variants of a carbohydrate-binding domain. While such fusions, binding, e.g., to cellulose or chitin, have been used to purify the fusion with the protein of interest on a carbohydrate column with subsequent cleavage of the partners (21, 22) or simply to immobilize proteins (23, 24), in our approach the immobilized fusion is used to purify a ligand of the fusion. We have optimized the fusion construct and found as the best format single-chain Fv antibody fragments (scFv fragments) with two chitin-binding domains (CBDs) C-terminally fused. An *E. coli* crude extract containing the scFv–CBD fusion protein is passed over chitin beads, by which the fusion protein is selectively immobilized, obviating the need for its prior purification. We demonstrate this principle with scFv fragments of several different specificities. Purifications have been carried out without any detector using gravity flow columns in combination with a positive pressure manifold, allowing the parallel purification of milligram amounts of 24 different samples in 6 h, including column preparation with fresh antibodies.

We also used an antibody against the His tag. This column can be put in series to an immobilized metal ion affinity column (IMAC) (25, 26), thereby leading to a generic rapid protein purification for His-tagged proteins using two different purification principles on the same tag.

MATERIALS AND METHODS

Plasmid construction. The plasmids for the periplasmic expression of scFv–CBD fusion proteins (Fig. 1) are based on the pAK series (27). The dHLX part of pAK500 was replaced by a linker–CBD fragment via *Eco*RI and *Hin*dIII. The linker–CBD fragment was amplified out of the plasmid pTYB1 (New England Biolabs, Beverly, MA) using the primers CBD5′__*Avr*II and CBD3′__*Nhe*I. CBD5′__*Avr*II, 5′-CATCCGGAATTCGGCGGTGGCTCCGAAGGCGGTGGCAGCGAAGGTGGCGGCCTAGGCACCACAAATCCTGGTG-3′, encoding the linker sequence (SGAEFGGGSEGGGSEGGGLG) including an *Eco*RI and an *Avr*II site. CBD3′__*Nhe*I, 5′-GTACCCAAGCTTAGCTAGCTTGAAGCTGCCACAAG-3′, introduced a *Nhe*I site and a *Hin*dIII site. The *Eco*RI site was used to clone a mIgG3hinge-dHLX fragment (derived from pACKdHLX (28)) for dimerization (Fig. 1). The *Nhe*I site allowed cloning of a second CBD fragment also amplified out of pTYB1, including the linker sequence. The primers CBD5′__*Eco*RI, 5′-CCGGAATTCGCTAGCGGTGGCCTGACC-3′, and CDB3′__*Nhe*I were used to introduce *Nhe*I sites at both ends. The resulting plasmids were called pKB100__wt (one CBD), pKB100dHLX__wt (dimeric miniantibody—two CBDs), pKB100__wtilwt (tandem CBDs), and pKB100dHLX__wtilwt (dimeric miniantibody—two tandem CBDs). The ribosome-binding site (RBS) was exchanged for the stronger T7G10 RBS of pAK400 (27) and the gene for coexpression of Skp was introduced (29) resulting in the pKB200 series (Fig. 1). The exchange of cysteine at position 30 (amino acid 30 of the isolated CBD, according to the numbering

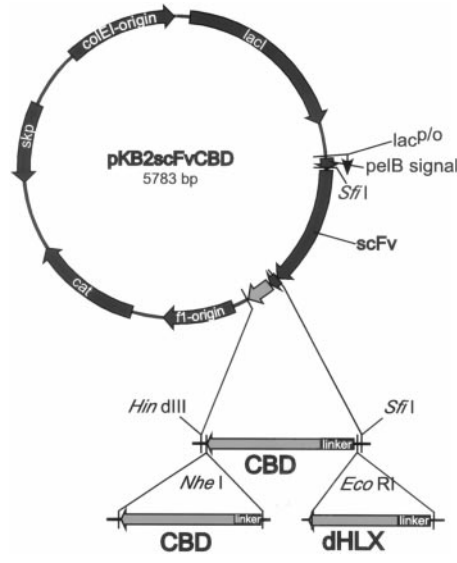

FIG. 1. Starting plasmid pKB2scFvCBD for cloning and expression of scFv–CBD fusion proteins. The plasmid is a derivative of the pAK series (27): The plasmid has a *lac* promoter and the strong RBS of pAK400 (27). Fusion proteins are expressed in the periplasm coexpressing the periplasmic folding factor Skp (29) regulated by its own promotor. Also represented are the restriction sites used for cloning of the dHLX fragment (28)—*Eco*RI—and for the second linker–CBD fragment—*Nhe*I.

of NEBs pTYB vector series; amino acid 677 in whole chitinase A1, see PDB code 1ED7 (30)) to serine and to alanine was performed by site-directed mutagenesis of all CBD fragments. The gene for the anti-His tag scFv fragment (mut12) (M. Kaufmann *et al.*, manuscript in press) was cloned as *Sfi*I cassette replacing the tet-resistance cassette. The scFv fragments directed against FkpA (6B1, 7B2, 9B3, and 11B4) as well as the scFvs K14G2 (anti-gpD scFv) and N7A9 (anti-SHP scFv) were selected out of the human combinatorial antibody library (HuCAL) (12) by two rounds of automated phage display (31). Nonsymmetrical *Sfi*I sites (27) were attached to the scFv cassettes of the HuCAL series by PCR and cloned into a pKB200 derivative containing gIIIpss (27) as stuffer, necessary to supply the *Eco*RI site. All HuCAL scFvs described here were introduced as *Mfe*I/*Eco*RI fragments, thus removing the second *Sfi*I site. The stuffer fragment was then exchanged for the CBDilCBD cassette by *Eco*RI/*Hin*dIII cloning (Fig. 1).

Expression of scFv–CBD fusions. The plasmids encoding the 12 different anti-His tag–CBD fusion proteins (Figs. 2 and 3; pKB2Hmut12 series, see Fig. 1) were transformed in the *E. coli* K12 strain SB536 (32) (F^-, WG1, Δ*fhu*A (*ton*A), Δ*hho*AB (SacII), *shh*). Small-scale expressions were performed at 25°C using 50 ml of SB medium (20 g/liter tryptone, 10 g/liter yeast extract, 5 g/liter NaCl, 50 mM K_2HPO_4) containing 30 μg/ml chloramphenicol. Cultures were inoculated from a 5-ml preculture to $OD_{550} = 0.1$. Expression was induced with 1 mM IPTG at an OD_{550} between 1.0 and 1.5. Cells were harvested 3 h after induction by centrifugation. Cell pellets were resuspended in MBS buffer (20 mM Mes/NaOH, pH 6.5, 500 mM NaCl, 0.1 mM EDTA), normalized to their end OD_{550} using 2.5 ml of MBS per 1 unit OD_{550}. Whole cell extracts were prepared by French press lysis at 10,000 psi and 1 ml of crude extract was centrifuged in an Eppendorf tube for 60 min at maximum speed and 4°C. The supernatants containing the soluble material and the pellets were analyzed in an anti-FLAG blot. Large-scale expressions of the scFv–CBD fusion proteins were carried out in culture volumes from 750 ml to 1 liter in 5-liter baffled shake flasks as described for the small cultures.

Expression of antigens. The expression of FkpA (33), GpHD (34), GpHDL-cCrk (34), and SHP (35) was carried out exactly following published protocols. His-tagged proteins (scFv 4D5–His, GroES–His, PhoA–His, CS–His, and GFP–His) were expressed in the *E. coli* strain JM83 (F^-, *ara*1, Δ(*lac-pro*AB), *rps*L (Str^R), *thi*1, φ80, Δ(*lac*Z)M15). Cultures (1 liter) were grown at 25°C in 5-liter baffled shake flasks using dYT medium (16 g/liter tryptone, 10 g/liter yeast extract, 5 g/liter NaCl) and were induced at $OD_{550} = 1$ with 1 mM IPTG (36). Cells were harvested 5 h after induction.

Batch binding experiment. Cell pellets of the anti-His tag fusion proteins were resuspended in TBST buffer (50 mM Tris/HCl, pH 8, 1 M NaCl, 0.1 mM EDTA, 1% Triton X-100) using 1 ml of buffer per 1 g of cells. DNase I (Roche Diagnostics, Basel, Switzerland) was added and cell disruption was achieved by French press lysis. After centrifugation (Sorvall SS34, 20,000 rpm, 4°C, 30 min) the supernatant was passed through a 0.22-μm filter (Millipore, Volketswil, Switzerland). To compensate for differences in expression levels of the constructs, estimated from Western blot analysis, crude extracts were diluted with TBST buffer to different extents. For the binding experiment those diluted crude extracts, now containing the same amount of fusion protein, were shaken with equilibrated chitin beads (New England Biolabs). Equilibrated chitin beads were prepared by using 100 μl of beads in an ethanol suspension and washing them three times with 1 ml of TBST buffer in a 2-ml Eppendorf tube. To these beads 500 μl of diluted crude *E. coli* extract containing the scFv–CBD fusion proteins was added. The mixture was shaken at 4°C for 1 h. The beads were washed three times with 1 ml of TBST buffer. One aliquot was taken and analyzed by SDS–PAGE to ensure that the same amount of each fusion protein had bound to the beads. The dissociation from the beads was determined by shaking the beads in 200 μl elution buffer (50 mM 3-[cyclohexylamino]-1-propane-sulfonic acid caps) (Sigma, Buchs, Switzerland)/NaOH, pH 10, 500 mM NaCl, 0.1 mM EDTA) for 1 h at 4°C. The beads were sedimented and the supernatant containing dissociated molecules was analyzed by an anti-FLAG blot.

SDS–PAGE and Western blot analysis. SDS–PAGE analyses were carried out under reducing conditions according to standard protocols using 12 and 15% polyacrylamide gels. Western blots with the monoclonal anti-FLAG antibody M1 were carried out as described (37, 38).

Preparation of crude extracts for chromatography experiments. Cell pellets expressing the fusion proteins (scFv–C30A–C30A constructs) used for chromatography experiments were resuspended in TBST buffer using 5 ml of buffer per 1 g of cells. After addition of DNase I, cell disruption was achieved by French press lysis. The suspension was clarified by centrifugation at maximum speed for 60 min and 4°C (Sorvall, SS34) and filtration through a 0.22-μm filter. The same procedure was performed with antigen cell pellets, but using MBS buffer (20 mM Mes/NaOH, pH 6.5, 500 mM NaCl, 0.1 mM EDTA).

Column chromatography. Chromatography experiments were performed using SPE columns, usually mounted in a positive pressure manifold (Fig. 4) (Varian Analytical Instruments Inc., Walnut Creek, CA). Empty SPE columns (Varian Bond Elut LRC, 10-ml columns)

were filled with 1.5 ml ethanol suspension of chitin beads (corresponding to approximately 1 ml settled beads) and after sedimentation a porous PTFE filter disk was placed on top of the settled column bed. Columns were equilibrated with 20 ml of TBST buffer. For equilibration a pressure of 0.4 bar was applied to the columns. Next, between 1.5 and 6 ml of scFv–C30A–C30A crude extract was loaded containing between 0.4 and 2 mg of fusion protein. No pressure was applied to the columns during loading of CBD fusions containing crude extracts. The columns were washed at 0.2 bar with 2 ml (2 column vol, cv) of TBST buffer and equilibrated with 8 ml (8 cv) of MBS buffer also at 0.2 bar. In the next step 2–3 ml crude extract containing the antigen was loaded without applying any pressure. The columns were washed with 20 ml (20 cv) MBS at 0.2 bar. For the anti-FkpA fusions an additional washing step at pH 3.2 (100 mM glycine/HCl, pH 3.2, 500 mM NaCl, 0.1 mM EDTA; 10 ml of buffer at 0.2 bar) was included (see Results). Elution of the different antigens was performed with 2 ml of elution buffer as follows: Elution from the anti-His tag columns could be achieved at pH 10 (50 mM CAPS/NaOH, pH 10, 500 mM NaCl, 0.1 mM EDTA). All four different anti-FkpA columns were eluted at pH 2 (100 mM glycine/HCl, pH 2, 500 mM NaCl, 0.1 mM EDTA). SHP could be either eluted at pH 3.2 or pH 10, while the elution of GpD or GpD fusion proteins was more effective at pH 3.2. Eluted fractions at pH 3.2 were neutralized with 17 μl 1 M Tris per milliliter, for the fractions eluted at pH 2 100 μl of 1 M Tris was required per milliliter.

Immobilized metal ion affinity chromatography. For the second purification step of the His-tagged proteins Ni–NTA Superflow (Qiagen, Basel, Switzerland) was used. As for the IAC runs, all IMAC experiments were performed using the positive pressure manifold. The reservoirs were filled with 0.5 ml sedimented Ni–NTA material. The gel bed was covered with a PTFE disc and equilibrated with 20 ml of binding buffer (50 mM Tris/HCl, pH 8, 500 mM NaCl, 20 mM imidazole). The pH 10-eluted fractions of the anti-His tag affinity columns were directly loaded. The columns were washed with 20 ml of binding buffer. Elutions were carried out with 2 ml of elution buffer (50 mM Tris/HCl, pH 8, 500 mM NaCl, 200 mM imidazole).

RESULTS

Construction and expression of CBD fusion proteins. The CBD (21, 30, 39) of *Bacillus circulans* WL-12 chitinase A1 (SwissProt No. P20533) was used. Preliminary tests of a fusion consisting of one CBD and a mutated version of the anti-His tag scFv 3D5 (36) showed bleeding from the column, suggesting that the binding of one CBD to chitin must be very weak. This observation is in contrast to expectations raised in the literature (21–24) (see also New England BioLabs' Impact T7 kit instruction manual), but consistent with quantitative determinations of the affinity of a cellulose binding domain from *Trichoderma reesei* to cellulose (40, 41). Consequently, stable binding to the beads, which is observed under many conditions, relies on the high molar concentration of chitin on the beads and is dynamic in nature (41, 42).

We therefore investigated the use of fusion proteins having more than one CBD. We fused two CBDs in tandem, dimerized the scFv to a miniantibody CBD fusion, or combined both strategies to generate a protein with four CBDs (Fig. 2). While all of these constructs showed better binding (Fig. 3B, wt constructs), periplasmic expression was decreased compared to the original scFv–CBD construct (Fig. 3A, wt constructs). As the CBD used contains a single unpaired cysteine, the periplasmic production of the fusion proteins is impaired. We therefore replaced the cysteine residue by serine or alanine, and indeed both mutations lead to much better expression of all constructs possessing two or four CBDs. Two constructs (scFv–C30S–C30S and scFv–C30A–C30A) showed about the same expression level as the simple scFv–CBD fusion.

For their use as affinity ligands stable binding of the fusion proteins to the column material is decisive. We therefore investigated the dissociation of all constructs from the chitin beads under elution conditions (used for the anti-His tag scFv fragment). The same amount of fusion protein was bound to the beads for all constructs. After shaking the beads for 1 h under elution conditions, the supernatant containing the dissociated molecules was analyzed by an anti-FLAG blot (Fig. 3B). The results suggest that both mutations result in weaker binding of the single CBDs, but there is almost no dissociation detectable for the scFv–CBD–CBD and the miniAb–CBD–CBD fusion proteins. Even though miniantibody–CBD fusions contain two CBDs as do the scFv–CBD–CBD constructs, they showed more leakage from the chitin beads. This may be due to some dissociation of the miniantibody over time. Summarizing these results, the scFv–C30A–C30A and the scFv–C30S–C30S fusion proteins show the highest expression level and sufficiently stable binding to the column material. Based on homology to other non-cysteine-containing CBDs (30) we decided to use the scFv–C30A–C30A construct as affinity ligand for our experiments.

To demonstrate the general applicability of our method we cloned scFv–C30A–C30A fusion proteins of six additional scFv fragments. All of them were selected out of the HuCAL (12), a naïve fully synthetic scFv library with a diversity of 2×10^9, by two rounds of phage display (31). Four scFv fragments with different frameworks for V_H and V_L as well as different CDR3s were selected as recognizing the *E. coli* protein FkpA (33, 43). Using similar procedures, a scFv fragment

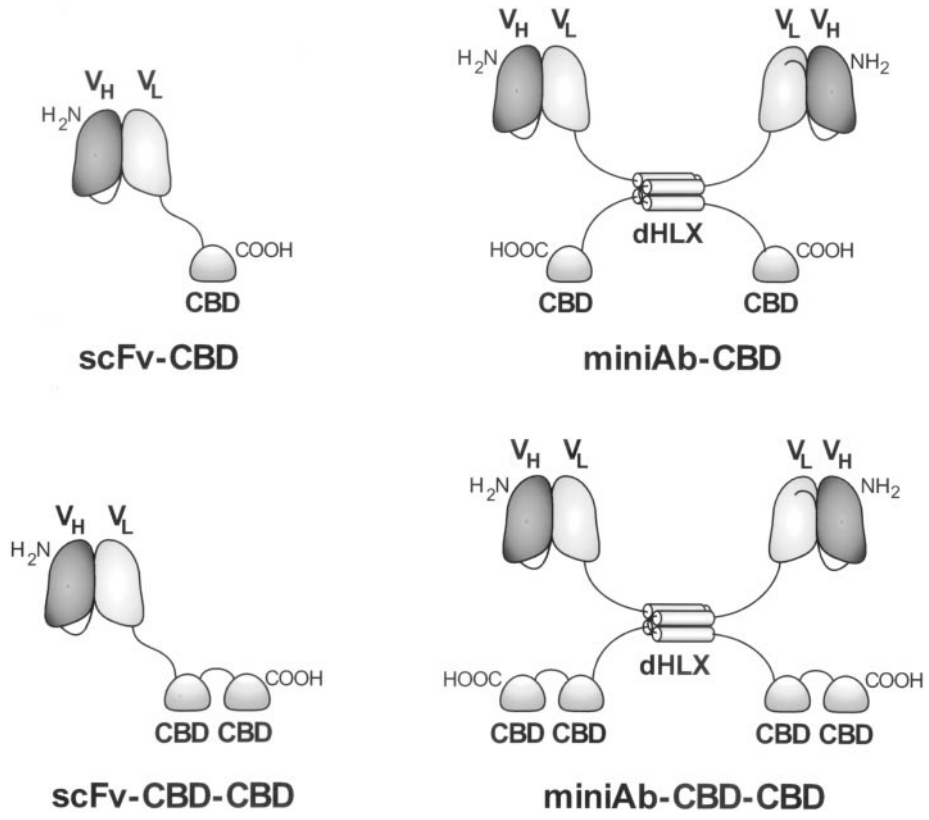

FIG. 2. Schematic representation of the fusion proteins containing one, two, or four CDBs. Each of the constructs was cloned with wt-CBDs and with CBDs, where the single cysteine was mutated to serine (C30S) or alanine (C30A), resulting in 12 different constructs. The scFv–CBD constructs have one CBD, while the dimeric molecules miniAb–CBD bind via two CBDs. The scFv–CBD–CBD constructs represent a tandem CBD. The combination of the dimerization motif and the tandem CBDs results in functional units possessing four CBDs, "miniantibodies, miniAbs."

specifically recognizing either the λ-phage coat protein gpD (34, 35) or the homologous protein SHP from phage 21 were selected.

Affinity purification. Most of the chromatography experiments were carried out using a positive pressure manifold (Fig. 4). Sample processing without any detector became possible after some preliminary tests to determine buffer volumes necessary for washing and elution of the columns. The low overpressure applied to the columns lead to a more uniform flow rate than under gravity flow.

To demonstrate the performance of the immobilized scFv–C30A–C30A fusion proteins as affinity ligands we show three different series of chromatography experiments. One series demonstrates purifications with the anti-gpD and anti-SHP ligands (Fig. 5A). gpD by itself and the his tagged kinase fusion protein gpHD-cCrk (34) bind to the anti-gpD column and can be eluted either at pH 3.2 or pH 10, and for this scFv fragment elution at pH 3.2 is more effective. SHP binds to its cognate scFv fragment and can be eluted at pH 3.2 and pH 10 with the same recovery.

In a second series of experiments we investigated the performance of four different anti-FkpA scFv fragments 6B1, 7B2, 9B3, and 11B4. The screening for elution conditions showed that elution of the antigen was only possible at pH 2, independent of the scFv immobilized. This tight binding made it possible to introduce an additional washing step at pH 3.2, removing some further contaminants. We immobilized the same defined amount of the scFv fusion proteins (0.4 mg) on the columns and overloaded them with FkpA. After washing the columns we could elute most FkpA from the 7B2 and 11B4 columns. While there was one additional band visible when the 11B4 scFv fragment was used all other columns yielded pure protein (Fig. 5B). The experiments in Figs. 5A and 5B show that the antibody itself is the most crucial determinant of purification quality and that with suitable antibodies, highly pure protein is obtained and no significant contaminants are introduced by the immobilization strategy.

The above examples have been carried out to investigate the range of applications of the immobilization concept described and the influence of the scFv chosen and to demonstrate that IAC can be easily standardized for parallel purification of different samples. A model

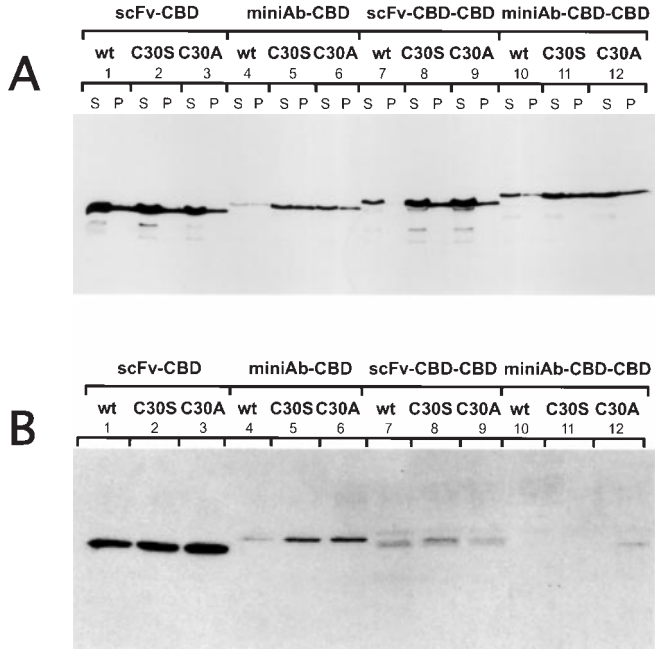

FIG. 3. Anti-FLAG blot showing the different expression levels and binding characteristics of the 12 different anti-His tag scFv–CBD fusion proteins. (A) Comparison of the expression level. S, soluble fraction of the fusion protein; P, pellet fraction. (B) Binding of the different constructs to chitin beads. Blots detecting the dissociated molecules are shown. Constructs suited as affinity ligands should therefore contain no band or only a weak band in the supernatant shown here. An independent blot (not shown) had verified that equal amounts of the constructs were initially incubated with the beads. Stable binding was found for all scFv–CBD–CBD and all miniAb–CBD–CBD constructs. The bands of dissociated protein that are visible in the corresponding lanes 7–12 are proteolytic digestion products carrying only one CBD (of the size of the molecules in Fig. 2), as can also be seen from Fig. 3A.

system—anti-His tag scFv and His-tagged GFP (44)—has been used to develop the protocol at different scales and in particular optimize the buffers applied for the purifications described above. Binding experiments with the anti-His tag–C30A–C30A fusion protein in different buffers showed that the fusion proteins bound completely in most buffers, including pH values between 3 and 10, NaCl concentrations between 0 and 1 M, and some additives such as 1% Triton X-100, 0.4 M arginine, 0.1% SDS. These results are in accordance with results described in the literature (21) and suggest that the equilibrium binding constant is not influenced much by the buffer components mentioned above. As the CBD is a very hydrophobic domain (30), membrane components associated with the CBDs initially clogged the columns and appeared as contaminants in the eluted fractions. This problem could be reduced to a minimum by addition of 1% Triton X-100 to the buffer. Additionally, a high concentration of NaCl (>1 M) reduces unspecific ionic interactions. In conclusion, TBST buffer (see Experimental Protocol) was found as the optimal binding buffer for all scFv–C30A–C30A fusion proteins. Further experiments showed that in the antigen loading step Triton X-100 was not necessary and that 500 mM NaCl was sufficient to reduce protein binding to chitin.

We then determined the capacity of the column for scFv–C30A–C30A fusion proteins and for the desired antigen. Batch binding experiments suggested that the capacity of chitin for CBD fusions is extremely high. Indeed, 100 μl of sedimented chitin beads bound approximately 5 mg of the anti-His tag–C30A–C30A fusion protein. Nevertheless, dynamic conditions are different, since some binding sites will not be accessible under flow conditions because of diffusion limitation, and bound molecules will dissociate from their binding sites and will be transported by the buffer flow resulting in a slow migration of the ligands over the column. Therefore, we recommend not to load more than 2 mg of scFv–C30A–C30A fusion proteins per 1 ml of settled chitin beads. This "dynamic capacity" is still in the upper range described for conventional affinity columns with monoclonal antibodies (2). To determine the capacity of antigen we carried out batch binding experiments with the GFP/anti-His tag model system, which demonstrated that the ratio of bound GFP to the scFv–C30A–C30A fusion is approximately 1:1 (data not shown).

Taking the above results together, it will generally be possible to purify on the order of 2 mg of the desired antigen with one of the 24 columns used in the positive pressure manifold. The procedure can be scaled up by

FIG. 4. Positive pressure manifold used for running of the affinity columns. Originally this device was designed for use in solid-phase extractions. With the "Cerex" model from Varian Inc. (Varian Analytical Instruments Inc., Walnut Creek, CA) the pressure applied on the columns can be adjusted very precisely to the low pressures required. This makes it possible to use the device for running affinity chromatography columns, where slow but even flow rates are required.

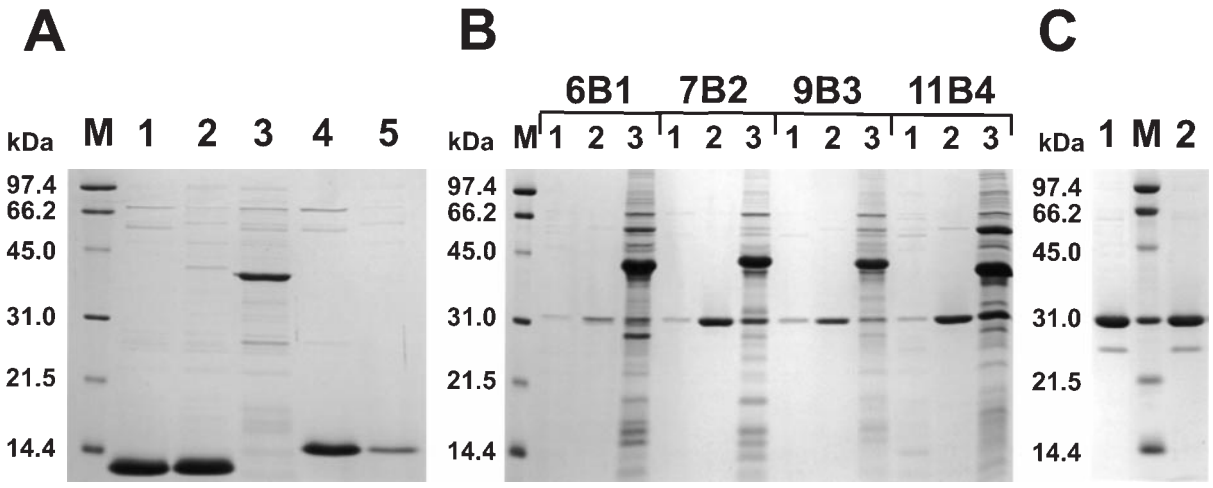

FIG. 5. SDS–PAGE analysis demonstrating the performance of purifications with different antibodies and elution conditions. The gels (15%) were run under reducing conditions and stained with Coomassie brilliant blue R-250. (A) Eluted fractions of the anti-gpD and anti-SHP affinity columns: (lane M) molecular weight marker, (lane 1) SHP eluted at pH 3.2, (lane 2) SHP eluted at pH 10, (lane 3) His-tagged gpD (gpHD) fusion protein eluted at pH 3.2 (gpHDL-cCrk), (lane 4) gpHD eluted at pH 3.2, and (lane 5) gpHD eluted at pH 10. (B). Purification of FkpA with four different scFv fragments 6B1, 7B2, 9B3, and 11B4. (Lane M) Molecular weight marker, (lane 1) wash at pH 3.2 where some further contaminants can be eluted, (lane 2) elution at pH 2, and (lane 3) affinity beads after the elution of bound FkpA. The affinity bead fractions show that approximately the same amount of each scFv fragment–CBD fusion (ca. 40.5 kDa) was bound to the column. Therefore, the different amounts of eluted FkpA are the result of different binding characteristics of the scFv fragments. (C) Purification of His-tagged GFP at different scales. (Lane 1) Small scale (2 mg) using the positive pressure manifold and (lane 2) large-scale purification, where approximately 20 mg have been purified using a FPLC system. Both purifications show the same degree of purity. The smaller band observed in the GFP–His purification (about 25 kDa) is a N-terminal proteolytic digestion product of GFP, still containing the C-terminal His tag.

running several identical columns on the manifold in parallel, by scaling up the gravity columns themselves (up to 5 ml) or by using conventional chromatographic equipment with pumps. We purified 20 mg His-tagged GFP on 17 ml beads with 27 mg scFv fusion (Fig. 5C). This procedure should be directly scalable to multigram amounts with any scFv fragment.

Two-step purification of His-tagged proteins. The above results demonstrate that IAC with the scFv fragments described could provide a tool for the one-step purification of the respective antigen to a high degree of purity (Fig. 5) which might be sufficient for many applications. However, we also wanted to provide a generic technology to obtain high purity proteins with as simple a procedure as plasmid purification. We thus developed a coupled anti-His tag/IMAC procedure in which the same tag is recognized twice but by totally different physical principles, and different contaminants should thus be removed. To avoid any dialysis steps and allow direct coupling of the columns we investigated elution conditions without imidazole for the anti-His tag affinity column and found that elution of His-tagged GFP was complete at pH 10 and resulted in a sharp peak. This convenient elution behavior is the result of this scFv fragment recognizing a protonated, C-terminally located His tag (36) which becomes deprotonated when the pH value is increased. To demonstrate the generality of the method we tested the following proteins: GroES–His (36, 45) (seven His tags), the scFv fragment 4D5–His (46) (one His tag), citrate synthase (36) (CS–His, two His tags), GFP–His (one His tag), and *E. coli* alkaline phosphatase (47) (PhoA–His, two His tags). Even though there are a different numbers of His tags on the different antigens, we could not detect any difference in the elution behavior of the antigens, as most antigen was found in the first 2-ml fraction of the elution (data not shown). The eluted fractions again show a very high degree of purity (Fig. 6, lanes A). They were directly loaded on the Ni–NTA column equilibrated with binding buffer containing 20 mM imidazole to reduce unspecific binding. With the coupled IMAC step we could clearly further reduce the contaminants (Fig. 6, lanes B). In conclusion, this two-step method, which uses a positive pressure manifold for both columns, is a valuable tool for the generic, easy, and fast purification of many His-tagged proteins in parallel on milligram scales.

DISCUSSION

In this work we present a novel strategy for the directed immobilization of recombinant antibody scFv fragments. Fusions with cysteine-free tandem CBDs were designed for high expression level and tight binding to the chitin matrix. The migration of the tandem

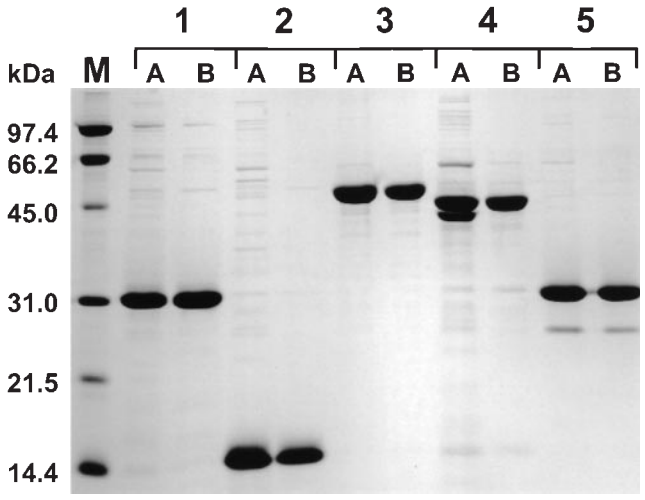

FIG. 6. Two-step purification of different His-tagged proteins. Lanes A, the eluted fractions of the anti-His tag affinity column; lanes B, the fractions after the anti-His tag affinity column coupled with IMAC. (Lane M) Molecular weight marker, (lane 1) scFv 4D5–His, (lane 2) GroES–His, (lane 3) PhoA–His, (lane 4) CS–His, and (lane 5) GFP–His.

CBD fusions over the column is very slow and the equilibrium of chitin-bound and dissociated CBD fusions is not shifted by the elution conditions, which leads to a selective dissociation of the antigen from the scFv fragment. Our results with seven different scFv fragments used to purify nine antigens demonstrate that this concept can be generally applied for IAC and yields highly pure protein when a suitable scFv fragment is used.

Compared to affinity chromatography methods using peptides (48) or other scaffolds (49) as ligands or to conventional IAC applications with mAbs our method has several advantages: (i) Instead of mAbs, whose production is expensive and time-consuming, we use scFv fragments which take advantage of modern library technologies and can be easily expressed in *E. coli* in large amounts. (ii) As the scFv fusion protein is coupled via the biological affinities of the CBD domains it is not involved in the immobilization procedure. This means that in contrast to conventional methods (1, 15, 16) no loss in binding capacity occurs. (iii) The use of random chemical coupling requires expensive activated matrices and purified ligands. Our method uses nonactivated carbohydrates, where the scFv fusion proteins bind selectively without the need for prior purification. (iv) Easy standardization and little instrumentation during the purifications make parallel sample processing possible.

There are several approaches for immobilizing recombinant antibody fragments reported in the literature. Most of them use chemically activated matrices (14–17), with all the problems described above for mAbs. Streptavidin matrices (19, 20), which are rather expensive, as streptavidin itself must be purified and chemically coupled to an activated matrix, have been used either with a tag which is biotinylated *in vivo*, but only 15% of the Fab fragment was biotinylated, leading to a low yield of immobilized molecules. If instead a weak-binding purely peptidic tag (Strep tag) is used (19), more functional molecules can be immobilized, but the whole complex of Fv fusion protein and antigen is eluted by biotin, thus requiring a second purification step. In contrast, our method yields almost 100% active immobilized molecules that remain bound on the column while the antigen is eluted.

Our concept should also be useful for therapeutic proteins, which normally must not carry a tag. Even though IAC with mAbs would provide a powerful purification, its use on the large scale required is economically prohibitive, and possible viral and prion contaminants introduced from the eukaryotic mAb production are another regulatory concern. The easy availability of scFv fragments from libraries and the inexpensive scalable technology described here makes this an attractive option for the first capturing step. Similarly, the simultaneous purification of untagged natural proteins for proteomics can make use of the same technology on a very small scale.

Finally, our method is also useful for His-tagged proteins. In many cases, His-tagged proteins are not pure enough after IMAC for the desired application and require a second step. Parallel purification has been implemented on small scales (50), but the enzymatic activity studies did not depend on having very pure samples. To maximize purification, gradients, and UV detectors are needed in IMAC, making parallel purification on milligram scales impractical. Here we use two columns in series which both recognize the His tag but with different physical principles. Since the eluant of the anti-His scFv column can be directly loaded onto the IMAC column and since no gradients or detectors are necessary, a very efficient and generic parallel protein purification is possible, inspired by modern parallel plasmid purification. The concept can be generalized from scFv proteins to any other protein with an affinity for the target molecule. Furthermore, this concept may also be useful to provide a capture surface for protein chip technology.

We believe that this technology may be of great value in the purification of proteins from small scales to large scales and may finally lead to a generic protein purification.

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REFERENCES

1. Cutler, P. (1996) Affinity chromatography. *Methods Mol. Biol.* 59, 157–168.
2. Jack, G. W., and Beer, D. J. (1996) Immunoaffinity chromatography. *Methods Mol. Biol.* 59, 187–196.
3. Jones, C., Patel, A., Griffin, S., Martin, J., Young, P., O'Donnell, K., Silverman, C., Porter, T., and Chaiken, I. (1995) Current trends in molecular recognition and bioseparation. *J. Chromatogr. A* 707, 3–22.
4. Sii, D., and Sadana, A. (1991) Bioseparation using affinity techniques. *J. Biotechnol.* 19, 83–98.
5. Ford, C. F., Suominen, I., and Glatz, C. E. (1991) Fusion tails for the recovery and purification of recombinant proteins. *Protein Express. Purif.* 2, 95–107.
6. Nilsson, J., Ståhl, S., Lundeberg, J., Uhlén, M., and Nygren, P. A. (1997) Affinity fusion strategies for detection, purification, and immobilization of recombinant proteins. *Protein Express. Purif.* 11, 1–16.
7. Smith, G. P., and Scott, J. K. (1993) Libraries of peptides and proteins displayed on filamentous phage. *Methods Enzymol.* 217, 228–257.
8. Winter, G., Griffiths, A. D., Hawkins, R. E., and Hoogenboom, H. R. (1994) Making antibodies by phage display technology. *Annu. Rev. Immunol.* 12, 433–455.
9. Hanes, J., Schaffitzel, C., Knappik, A., and Plückthun, A. (2000) Picomolar affinity antibodies from a fully synthetic naïve library selected and evolved by ribosome display. *Nature Biotechnol.* 18, 1287–1292.
10. Plückthun, A., Schaffitzel, C., Hanes, J., and Jermutus, L. (2000) *In vitro* selection and evolution of proteins. *Adv. Protein Chem.* 55, 367–403.
11. Mössner, E., Koch, H., and Plückthun, A. (2001) Fast selection of antibodies without antigen purification: Adaptation of the protein fragment complementation assay to select antigen-antibody pairs. *J. Mol. Biol.* 308, 115–122.
12. Knappik, A., Ge, L., Honegger, A., Pack, P., Fischer, M., Wellnhofer, G., Hoess, A., Wolle, J., Plückthun, A., and Virnekäs, B. (2000) Fully synthetic human combinatorial antibody libraries (HuCAL) based on modular consensus frameworks and CDRs randomized with trinucleotides. *J. Mol. Biol.* 296, 57–86.
13. Vaughan, T. J., Williams, A. J., Pritchard, K., Osbourn, J. K., Pope, A. R., Earnshaw, J. C., McCafferty, J., Hodits, R. A., Wilton, J., and Johnson, K. S. (1996) Human antibodies with sub-nanomolar affinities isolated from a large non-immunized phage display library. *Nature Biotechnol.* 14, 309–314.
14. Arnold-Schild, D., Kleist, C., Welschof, M., Opelz, G., Rammensee, H. G., Schild, H., and Terness, P. (2000) One-step single-chain Fv recombinant antibody-based purification of gp96 for vaccine development. *Cancer Res.* 60, 4175–4178.
15. Berry, M. J., Davies, J., Smith, C. G., and Smith, I. (1991) Immobilization of Fv antibody fragments on porous silica and their utility in affinity chromatography. *J. Chromatogr.* 587, 161–169.
16. Berry, M. J., and Pierce, J. J. (1993) Stability of immunoadsorbents comprising antibody fragments: Comparison of Fv fragments and single-chain Fv fragments. *J. Chromatogr.* 629, 161–168.
17. Molloy, P., Brydon, L., Porter, A. J., and Harris, W. J. (1995) Separation and concentration of bacteria with immobilized antibody fragments. *J. Appl. Bacteriol.* 78, 359–365.
18. Turkova, J. (1999) Oriented immobilization of biologically active proteins as a tool for revealing protein interactions and function. *J. Chromatogr. B Biomed. Sci. Appl.* 722, 11–31.
19. Kleymann, G., Ostermeier, C., Ludwig, B., Skerra, A., and Michel, H. (1995) Engineered Fv fragments as a tool for the one-step purification of integral multisubunit membrane protein complexes. *Bio/Technology (N. Y.)* 13, 155–160.
20. Weiss, E., Chatellier, J., and Orfanoudakis, G. (1994) *In vivo* biotinylated recombinant antibodies: construction, characterization, and application of a bifunctional Fab-BCCP fusion protein produced in *Escherichia coli*. *Protein Express. Purif.* 5, 509–517.
21. Chong, S., Mersha, F. B., Comb, D. G., Scott, M. E., Landry, D., Vence, L. M., Perler, F. B., Benner, J., Kucera, R. B., Hirvonen, C. A., Pelletier, J. J., Paulus, H., and Xu, M. Q. (1997) Single-column purification of free recombinant proteins using a self-cleavable affinity tag derived from a protein splicing element. *Gene* 192, 271–281.
22. Shpigel, E., Elias, D., Cohen, I. R., and Shoseyov, O. (1998) Production and purification of a recombinant human hsp60 epitope using the cellulose-binding domain in *Escherichia coli*. *Protein Express. Purif.* 14, 185–191.
23. Ramirez, C., Fung, J., Miller, R. C., Jr., Antony, R., Warren, J., and Kilburn, D. G. (1993) A bifunctional affinity linker to couple antibodies to cellulose. *Bio/Technology (N. Y.)* 11, 1570–1573.
24. Shpigel, E., Goldlust, A., Efroni, G., Avraham, A., Eshel, A., Dekel, M., and Shoseyov, O. (1999) Immobilization of recombinant heparinase I fused to cellulose-binding domain. *Biotechnol. Bioeng.* 65, 17–23.
25. Hochuli, E., Bannwarth, W., Döbeli, H., Gentz, R., and Stüber, D. (1988) Genetic approach to facilitate purification of recombinant proteins with a novel metal chelate adsorbent. *Bio/Technology (N. Y.)* 6, 1321–1325.
26. Lindner, P., Guth, B., Wülfing, C., Krebber, C., Steipe, B., Müller, F., and Plückthun, A. (1992) Purification of native proteins from the cytoplasm and periplasm of *Escherichia coli* using IMAC and histidine tails: a comparison of proteins and protocols. *Methods: Companion Methods Enzymol.* 4, 41–56.
27. Krebber, A., Bornhauser, S., Burmester, J., Honegger, A., Willuda, J., Bosshard, H. R., and Plückthun, A. (1997) Reliable cloning of functional antibody variable domains from hybridomas and spleen cell repertoires employing a reengineered phage display system. *J. Immunol. Methods* 201, 35–55.
28. Pack, P., and Plückthun, A. (1992) Miniantibodies: use of amphipathic helices to produce functional, flexibly linked dimeric Fv fragments with high avidity in *Escherichia coli*. *Biochemistry* 31, 1579–1584.
29. Bothmann, H., and Plückthun, A. (1998) Selection for a periplasmic factor improving phage display and functional periplasmic expression. *Nature Biotechnol.* 16, 376–380.
30. Ikegami, T., Okada, T., Hashimoto, M., Seino, S., Watanabe, T., and Shirakawa, M. (2000) Solution structure of the chitin-binding domain of *Bacillus circulans* WL-12 chitinase A1. *J. Biol. Chem.* 275, 13654–13661.
31. Krebs, B., Rauchenberger, R., Reiffert, S., Rothe, C., Tesar, M., Thomassen, E., Cao, M., Dreier, T., Fischer, D., Höß, A., Inge, L., Knappik, A., Marget, M., Pack, P., Meng, X., Schier, R., Söhlemann, P., Winter, J., Wölle, J., and Kretzschmar, T. (2001) High-throughput generation and engineering of recombinant human antibodies. *J. Immunol. Methods* 254, 67–84.
32. Bass, S., Gu, Q., and Christen, A. (1996) Multicopy suppressors of prc mutant *Escherichia coli* include two HtrA (DegP) protease

homologs (HhoAB), DksA, and a truncated R1pA. *J. Bacteriol.* 178, 1154–1161.

33. Ramm, K., and Plückthun, A. (2000) The periplasmic *Escherichia coli* peptidylprolyl cis,trans-isomerase FkpA. II. Isomerase-independent chaperone activity *in vitro*. *J. Biol. Chem.* 275, 17106–17113.
34. Forrer, P., and Jaussi, R. (1998) High-level expression of soluble heterologous proteins in the cytoplasm of *Escherichia coli* by fusion to the bacteriophage lambda head protein D. *Gene* 224, 45–52.
35. Yang, F., Forrer, P., Dauter, Z., Conway, J. F., Cheng, N., Cerritelli, M. E., Steven, A. C., Plückthun, A., and Wlodawer, A. (2000) Novel fold and capsid-binding properties of the lambda-phage display platform protein gpD. *Nature Struct. Biol.* 7, 230–237.
36. Lindner, P., Bauer, K., Krebber, A., Nieba, L., Kremmer, E., Krebber, C., Honegger, A., Klinger, B., Mocikat, R., and Plückthun, A. (1997) Specific detection of his-tagged proteins with recombinant anti-His tag scFv-phosphatase or scFv-phage fusions. *Biotechniques* 22, 140–149.
37. Ge, L., Knappik, A., Pack, P., Freund, C., and Plückthun, A. (1995) *in* "Antibody Engineering" (Borrebaeck, C. A. K., Ed.), 2nd ed, pp. 229–266, Oxford Univ. Press, New York, NY.
38. Knappik, A., and Plückthun, A. (1994) An improved affinity tag based on the FLAG peptide for the detection and purification of recombinant antibody fragments. *Biotechniques* 17, 754–761.
39. Watanabe, T., Ito, Y., Yamada, T., Hashimoto, M., Sekine, S., and Tanaka, H. (1994) The roles of the C-terminal domain and type III domains of chitinase A1 from *Bacillus circulans* WL-12 in chitin degradation. *J. Bacteriol.* 176, 4465–4472.
40. Linder, M., Salovuori, I., Ruohonen, L., and Teeri, T. T. (1996) Characterization of a double cellulose-binding domain: Synergistic high affinity binding to crystalline cellulose. *J. Biol. Chem.* 271, 21268–21272.
41. Linder, M., Nevanen, T., Söderholm, L., Bengs, O., and Teeri, T. T. (1998) Improved immobilization of fusion proteins via cellulose-binding domains. *Biotechnol. Bioeng.* 60, 642–647.
42. Carrard, G., Koivula, A., Söderlund, H., and Beguin, P. (2000) Cellulose-binding domains promote hydrolysis of different sites on crystalline cellulose. *Proc. Natl. Acad. Sci. USA* 97, 10342–10347.
43. Bothmann, H., and Plückthun, A. (2000) The periplasmic *Escherichia coli* peptidylprolyl cis,trans-isomerase FkpA. I. Increased functional expression of antibody fragments with and without cis-prolines. *J. Biol. Chem.* 275, 17100–17105.
44. Crameri, A., Whitehorn, E. A., Tate, E., and Stemmer, W. P. (1996) Improved green fluorescent protein by molecular evolution using DNA shuffling. *Nature Biotechnol.* 14, 315–319.
45. Nieba, L., Nieba-Axmann, S. E., Persson, A., Hamalainen, M., Edebratt, F., Hansson, A., Lidholm, J., Magnusson, K., Karlsson, A. F., and Plückthun, A. (1997) BIACORE analysis of histidine-tagged proteins using a chelating NTA sensor chip. *Anal. Biochem.* 252, 217–228.
46. Carter, P., Kelley, R. F., Rodrigues, M. L., Snedecor, B., Covarrubias, M., Velligan, M. D., Wong, W. L. T., Rowland, A. M., Kotts, C. E., Carver, M. E., Yang, M., Bourell, J. H., Shepard, H. M., and Henner, D. (1992) High level *Escherichia coli* expression and production of a bivalent humanized antibody fragment. *Bio/Technology (N. Y.)* 10, 163–167.
47. Inouye, H., Michaelis, S., Wright, A., and Beckwith, J. (1981) Cloning and restriction mapping of the alkaline phosphatase structural gene (phoA) of *Escherichia coli* and generation of deletion mutants *in vitro*. *J. Bacteriol.* 146, 668–675.
48. Nord, K., Gunneriusson, E., Uhlén, M., and Nygren, P. A. (2000) Ligands selected from combinatorial libraries of protein A for use in affinity capture of apolipoprotein A-1_M and *Taq* DNA polymerase. *J. Biotechnol.* 80, 45–54.
49. Sproule, K., Morrill, P., Pearson, J. C., Burton, S. J., Hejnæs, K. R., Valore, H., Ludvigsen, S., and Lowe, C. R. (2000) New strategy for the design of ligands for the purification of pharmaceutical proteins by affinity chromatography. *J. Chromatogr. B Biomed. Sci. Appl.* 740, 17–33.
50. Lanio, T., Jeltsch, A., and Pingoud, A. (2000) Automated purification of His6-tagged proteins allows exhaustive screening of libraries generated by random mutagenesis. *Biotechniques* 29, 338–342.