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Purification of Plant-Derived Antibodies through Direct Immobilization of Affinity Ligands on Cellulose

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Plants possess enormous potential as factories for the large scale production of therapeutic reagents such as recombinant proteins and antibodies. A major factor limiting commercial advances of plant-derived pharmaceuticals is the cost and inefficiency of purification. As a model system, we have developed a simple yet robust method for immobilizing affinity capture ligands onto solid supports by interfacing the secreted expression and coupling of a chimeric fusion protein in *Pichia pastoris* to microcrystalline cellulose in a single step. The fusion protein, which consisted of antibody-binding proteins L and G fused to a cellulose-binding domain (LG-CBD), was tethered directly onto cellulose resins added to *P. pastoris* cultures and subsequently used for antibody purification. Both the antibody-binding protein L and protein G domains were functional, as demonstrated by the ability of cellulose-immobilized LG-CBD to purify both a scFv antibody fragment from yeast and a human IgG1 monoclonal antibody from transgenic tobacco. Furthermore, combining two *P. pastoris* strains expressing LG-CBD and scFv with CP-102 cellulose in a single culture allowed for easy recovery of biologically active scFv. Direct immobilization of affinity purification ligands, such as LG-CBD, onto inexpensive support matrices such as cellulose is an effective method for the generation of functional, single-use antibody purification reagents. Straightforward preparation of purification reagents will help make antibody purification from genetically modified crop plants feasible and address one of the major bottlenecks facing commercialization of plant-derived pharmaceuticals.

KEYWORDS: Antibody; cellulose-binding domain; coexpression; microcrystalline cellulose; purification; protein LG

INTRODUCTION

The production of pharmaceutical reagents (i.e., recombinant proteins, antibodies and vaccines) in plants is of considerable interest to both the agricultural and pharmaceutical industries. Recombinant protein-based pharmaceuticals produced in plants offer the advantages of large-scale production, ease of storage and distribution, and product safety compared to current production sources. In addition, commercialization of plant-derived pharmaceuticals could open new markets for farmers whose crops have traditionally been used for a single purpose (i.e., tobacco). Currently, one of the technical bottlenecks limiting commercial-scale production of plant-derived antibodies (Abs) and other bioproducts is the high cost and inefficiency of downstream processing and purification (*1*). Purification of monoclonal antibodies (mAbs) and Ab fragments from plants relies on commercial chromatography reagents such as Streamline protein A (*2*), protein A magnetic beads (*3*), expanded-bed adsorption-immobilized metal affinity chromatography (EBA-IMAC) (*4*, *5*), protein G-agarose (*6*), or combinations thereof (*7*). High levels of plant polysaccharides and phenolic compounds can interfere with purification (*8*, *9*), and from our experience poorly clarified plant homogenates can lead to resin fouling, requiring considerable maintenance time (J.C.H., unpublished data). As such, the development of cheap, robust, and single-use affinity ligand reagents is desirable for purification of bioproducts from plants and other complex feed stocks. Conceivably, costs may be reduced further if a plant could coexpress its own affinity purification ligand and the bioproduct of interest (*10*, *11*).

Cellulose is an attractive support matrix for affinity purification reagents because of its favorable biophysical properties, low cost, and low nonspecific affinity for most proteins (*12*). Over the years, several affinity ligands have been constructed based on the fusion of an antibody-binding domain (i.e., protein A, protein G, protein L) to a cellulose-binding domain (CBD). These fusion proteins include, but are not limited to, protein A-CBD (*12*−*15*), protein L-CBD (Fluka Chemical Co.) and LG-CBD (*16*). Originally identified in *Trichoderma reesei* and *Cellulomonas fimi*, CBDs are the noncatalytic domain of glycosidic hydrolase enzymes responsible for anchoring the enzyme on insoluble

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cellulose through high-affinity noncovalent interactions (*17*, *18*). Cellulose-binding domains are ideal immobilization domains for affinity ligands because they fold independently and do not interfere with their fusion partner (*11*); coupling to cellulose matrices orients the fusion partner away from the solid support (*16*) reducing steric hindrance; and their high-affinity binding to cellulose is considered nearly irreversible (*19*). At present, many CBD-tagged affinity ligands are purified before attachment to their solid support matrix (*12*, *16*). For large-scale applications, it could be beneficial to directly immobilize the affinity ligand at the source of production, thus avoiding the cost and time required for purification. A potential use of cellulose-supported affinity ligands is for the purification of bioproducts from complex feed stocks, such as homogenates from genetically modified plants expressing recombinant proteins such as mAbs and Ab fragments (*20*−*23*).

To examine the potential of immobilizing affinity purification ligands onto cellulose matrices in a single step, we engineered the yeast *P. pastoris* to express and secrete a chimeric protein consisting of antibody-binding proteins L and G (*24*) fused to a cellulose-binding domain (CBD2a) (*25*). While a similar fusion was recently reported for cell capture in hollow-fiber bioreactors (*16*), we explored the direct immobilization of LG-CBD onto cellulosic resins for antibody purification applications. Our chimeric fusion protein could bind cellulose resins added directly to yeast cultures, alleviating the need to purify the affinity ligand. Both protein L and protein G domains retained dual functionality as a fusion to CBD, as demonstrated by the specific binding and purification of scFv and IgG antibodies from complex feed stocks (i.e., yeast supernatants and tobacco plant homogenates). In addition, a yeast coexpression system involving a culture of two yeasts that expressed LG-CBD and scFv in the presence of cellulose resin was investigated, demonstrating rapid Ab fragment production and purification from a single culture in essentially one step. This is a first step toward the rapid generation of inexpensive affinity purification reagents and systems, to reduce the costs associated with downstream processing of pharmaceutical products, including antibodies, from complex production systems such as genetically modified crop plants.

MATERIALS AND METHODS

Cell Lines and Plasmids. TOP 10 F′ *Escherichia coli* cells (Invitrogen, Carlsbad, CA) were used for all cloning procedures. *P. pastoris* strain X-33 cells (Invitrogen) were used for recombinant protein expression. All cloning was performed in the pPICZαA expression vector (Invitrogen).

DNA Synthesis and Assembly of Expression Vectors. Nucleotides encoding a version of a family 2a cellulose-binding domain (formerly CBD_{Cex}), engineered to be devoid of 5 putative N-glycosylation sites (*25*), were codon-optimized for expression in *P. pastoris* by GeneArt (Regensburg, Germany). A 5′ *Xba*I site followed by a proline-threonine rich linker region (*26*) and a 3′ *Sal*I site were included during synthesis. The CBD DNA was subcloned into the vector pPICZαA via *Xba*I and *Sal*I restriction enzyme sites. The nucleotides encoding a modified version of the previously reported protein LG sequence (*24*) were codon-optimized for eukaryotic expression by incorporating preferred *P. pastoris* and tobacco codons. A putative N-glycosylation site was removed during DNA synthesis by substituting Asn30 → Asp30 (**Table 1**). A 5′ *Xho*I site followed by the *P. pastoris* Kex2/Ste13 signal cleavage site and a 3′ *Xba*I site were also included in the synthesized DNA. The DNA was subcloned into pPICZαA-CBD by *Xho*I and *Xba*I, creating pPICZαA-LG-CBD. This vector was transformed into chemically competent *E. coli* and plasmid was isolated for DNA sequencing prior to transformation of *P. pastoris* according to protocols provided by Invitrogen.

The DNA encoding an anti-group B *Streptococcus* (GBS) scFv (*27*) was cloned into pPICZαA for expression in *P. pastoris*. PCR primers (For 5′-TGTGATTCTGCTCGAGCGTAGAGAGGCTGAAGCTGAGGTGCAGCTGTTGGAGTCTGG-3′) and (Rev 5′-TTTGTTCTAGACTATCAAGCGTAATCTGGAACATCGTATGGGTATGCGGCCCCGTGATGGG-3′) were used to amplify anti-GBS scFv DNA and introduce a 5′ *Psp*XI restriction site and Ste13/Kex2 signal cleavage site, as well as a 3′ hemagglutinin epitope tag (HA) and a *Xba*I site. Amplified DNA was digested with *Psp*XI and *Xba*I and ligated into pPICZαA that was digested with *Xho*I and *Xba*I, creating pPICZαA-GBS-scFv. The vector was transformed into chemically competent *E. coli* and the plasmid isolated for DNA sequencing and transformation of *P. pastoris*.

Table 1. Amino Acid Substitutions Incorporated into LG-CBD To Prevent N-Linked Glycosylation

substitution	domain	ref
N30 → D	protein L	this study
N472 → Q	CBD	*25*
S479 → G	CBD	*25*
S523 → N	CBD	*25*
N535 → Q	CBD	*25*
T553 → A	CBD	*25*

Transformation of *P. pastoris*. Two plasmids (pPICZαA control and pPICZαA-LG-CBD) were linearized with *Pme*I (New England Biolabs, Mississauga, Canada), gel purified, and transformed into chemically competent *P. pastoris* cells (strain X-33) according to the manufacturer's instructions. The vector pPICZαA-GBS-scFv was linearized with *Sac*I (NEB), gel purified, and transformed. All *P. pastoris* clones were selected on yeast-peptone-dextrose (YPD) agar plates containing 100 μg/mL Zeocin antibiotic.

Protein Expression and Purification. Single colonies of *P. pastoris* transformed with pPICZαA, pPICZαA-LG-CBD or pPICZαA-GBS-scFv were used to inoculate 10 mL of BMGY medium (Invitrogen) and were grown at 30 °C with shaking at 250 rpm until OD_{600} = 0.6−1.0 (18 h). These 10 mL cultures were used to inoculate 240 mL of BMGY, which were grown to an OD_{600} = 0.6−1.0 (18−22 h) under the same conditions. To induce protein expression, the 250 mL BMGY cultures were centrifuged at 2,500 rpm for 10 min, the supernatant was decanted, and cell pellets were resuspended in 1 L of BMMY (Invitrogen) supplemented with 2% casamino acids in 4 L flasks. These cultures were grown for 96−120 h at 30 °C while shaking at 250 rpm and supplemented with 0.5% sterile methanol every 24 h. Recombinant LG-CBD was initially purified by immobilized metal-affinity chromatography (IMAC) using an AKTA FPLC (GE Healthcare, Piscataway, NJ) by loading filtered *P. pastoris* supernatants onto a 5 mL HisTrap column (GE Healthcare) and eluted with a stepwise imidazole gradient (0−500 nM). Purified LG-CBD eluted from the IMAC column was analyzed by SDS−PAGE, dialyzed into PBS pH 7.3 and stored at 4 °C.

Binding Capacity of LG-CBD for Cellulose Resins. The binding capacity of purified LG-CBD for Avicel PH-101 (Sigma, Oakville, Canada), Celphere SCP-101 (Asahi Kasei Chemical Co., Tokyo, Japan), and Celphere CP-102 (Asahi Kasei) microcrystalline cellulose resins was determined essentially as described (*14*). Briefly, a range of LG-CBD concentrations (5−1000 μg diluted in 1 mL of PBS) were incubated with 25 mg of each of the 3 microcrystalline cellulose resins for 3 h at 21 °C. Binding was determined by calculating the difference in absorbance measurements of LG-CBD (A_{280} nm, ε = 75750 M^{-1} cm^{-1}) before and after incubation with cellulose.

Direct Immobilization of LG-CBD onto Cellulose. Direct immobilization of LG-CBD onto cellulose resins during the growth of *P. pastoris* cell cultures involved supplementing 1 L cultures with 8 g of sterilized Avicel PH-101, Celphere SCP-101 or Celphere CP-102 microcrystalline cellulose resins. After 96−120 h, resins were allowed to settle and the *P. pastoris* culture supernatant was decanted. As only CP-102 resin had sufficient density for simple gravity-based separation of supernate, work with the other two resins was terminated. LG-CBD bound to the CP-102 resin was extensively washed (6 × 300 mL of PBS + 1% (v/v) Tween-20) and stored at 4 °C in the presence of 20% ethanol. To quantify the amount of LG-CBD immobilized on CP-102, two methods were used. First, LG-CBD was stripped from CP-102 (50 mg) by boiling for 10 min with SDS−PAGE sample buffer containing β-mercaptoethanol and compared to serially diluted standards on a 12% acrylamide SDS−PAGE gel. Second, LG-CBD was stripped from CP-102 by incubating ∼50 mg of resin with 0.5 mL of 1 M NaOH for 3 h and the absorbance read at A_{280}.

Purification of IgG from Transgenic Tobacco. Fresh tobacco leaves (125 g) were harvested from 4-month-old transgenic tobacco (*Nicotiana tabacum*, T_2 generation) expressing a fully human anti-*Pseudomonas* IgG1 antibody (*7*), homogenized in a food processor and resuspended in 75 mL of PBS containing 1% (v/v) Tween 20 (PBS-T). The tobacco homogenate was centrifuged at 12000*g* for 30 min (4 °C), after which the supernatant was recovered and centrifuged for an additional 30 min under the same conditions. Approximately 150 mL of recovered supernatant was added to a 300 mL beaker with 2 mL of affinity reagent (~1.3 g of CP-102 microcrystalline cellulose containing 1.2 mg of immobilized LG-CBD) and incubated with gentle stirring for 3 h at room temperature. The CP-102 + LG-CBD resin was allowed to settle on the bottom of the beaker and the tobacco supernatant decanted before washing with 4 × 200 mL of PBS-T. Washed CP-102 + LG-CBD was transferred to a 20 mL disposable column (1.5 × 14 cm; Bio-Rad, Hercules, CA) and washed with an additional 10 bed volumes of PBS-T. IgG antibodies were eluted from CP-102 + LG-CBD by 3 × 5 mL elutions of 100 mM glycine (pH 2.3) and immediately neutralized with 1 M Tris pH 7.5. A sample of LG-CBD was stripped from CP-102 (50 mg) after elution by boiling with SDS−PAGE sample buffer to confirm all IgG was eluted. All samples, including plant homogenate before and after purification, were analyzed by SDS−PAGE. Purified IgG concentrations were determined by absorbance readings at A_{280}. Purified IgG antibodies were analyzed by Western immunoblotting to confirm their presence. Wild-type tobacco plants (control) were processed in the same manner as described. In addition, 125 g of tobacco leaves expressing the anti-*Pseudomonas* IgG1 was purified using the IMAC + protein G-agarose method exactly as described (*7*).

To confirm that the binding of IgG1 to immobilized LG-CBD was specific, approximately 100 μg of anti-*Pseudomonas* IgG1 was passed over 1 mL of either CP-102 cellulose, protein L-agarose (MJS BioLynx, Brockville, Canada), protein G-agarose (GE Healthcare), or CP-102 + LG-CBD in a 10 mL BioRad column, washed, and eluted as indicated by the manufacturer's instructions or as noted above. Eluates were analyzed by SDS−PAGE under reducing conditions.

Functional Characterization of Purified IgG. Two enzyme-linked immunosorbent assays (ELISA) were performed to illustrate the functionality of purified anti-*Pseudomonas* IgG. In the first assay, three heat-killed strains of *P. aeruginosa* (O6ad, PA01 and PA10) and lipopolysaccharide (LPS) derived from the same three strains, prepared essentially as described (*7*), were used to determine the specificity of IgG purified from LG-CBD. Heat-killed bacteria (1×10^7 cfu/100 μL; 100 μL/well) or LPS (1 μg/mL; 100 μL/well) diluted in PBS pH 7.3, were coated in 96-well plates (Nalgene-Nunc, Rocherster, NY) for 16 h at 4 °C and blocked with 200 μL of blocking buffer (3% skim milk in PBS-T) for 1 h. Purified IgG (225 ng/well) diluted in PBS was added for 1 h and washed with PBS-T. Wells were incubated with HRP-labeled goat antihuman IgG (Sigma), diluted 1:2000 in PBS (100 μL/well), for 1 h followed by 3 PBS-T washes. TMB substrate (MJS BioLynx; 100 μL/well) was added, the reaction stopped by addition of 1.5 M H_2SO_4 (100 μL/well) and the absorbance read at 450 nm on a Bio-Rad plate reader (Bio-Rad). No coating antigen (i.e., no heat-killed bacteria or LPS) and no anti-*Pseudomonas* IgG served as reaction controls. In the second assay, heat-killed O6ad was used to compare differences in the activity of equivalent concentrations of IgG purified by either LG-CBD or by the (IMAC + protein G) method reported by McLean et al. (*7*). The assay was performed in the same manner as describe above, with the exception of purified anti-*Pseudomonas* IgG which was serially diluted from 125 ng/mL to 8 ng/mL.

Coexpression and Purification of scFv Antibodies from *P. pastoris*. Two strains of *P. pastoris*, one expressing LG-CBD and another expressing anti-GBS scFv, were used to inoculate 250 mL of BMMY expression media containing 2 g of CP-102 resin and grown for 96 h under the same conditions as describe previously for *P. pastoris* cell culture. Afterward, the resin was harvested by decanting the cell debris and extensive washing (4 × 300 mL) with PBS-T. Washed resin was transferred to a 20 mL disposable column (BioRad; 1.5 × 14 cm) and washed with an additional 10 bed volumes of PBS-T. Anti-GBS scFv was eluted from CP-102 + LG-CBD by 4 × 5 mL elutions of 100 mM glycine pH 2.0 and immediately neutralized with 1 M Tris pH 7.5. Each eluent (5 mL) was incubated on the column for 10 min. A sample of CP-102 (50 mg) containing LG-CBD was removed from the column before and after scFv elution and stripped by boiling at 100 °C for 10 min in SDS−PAGE sample buffer. These and eluted fractions were analyzed by SDS−PAGE. *P. pastoris* expressing anti-GBS scFv was grown in the presence of CP-102 resin alone as a nonspecific binding control.

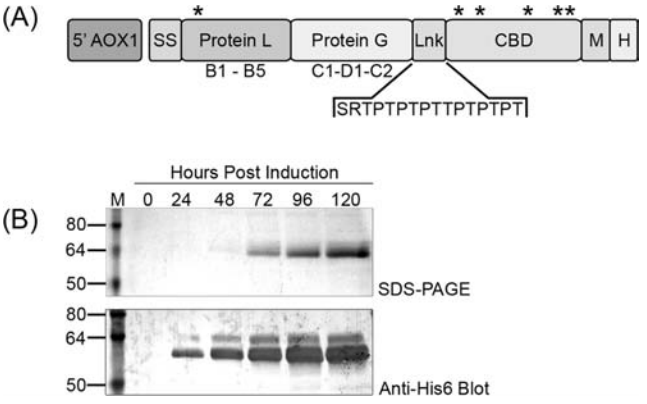

Figure 1. Assembly and expression of the trifunctional affinity ligand LG-CBD in *Pichia pastoris*. (**A**) Protein L (subunits B1−B5), protein G (subunits C1-D1-C2) and cellulose binding domain 2a (CBD2a) were assembled in the *P. pastoris* expression vector pPICZαA. A 16-mer linker between the protein G and CBD domains is shown. Expression of LG-CBD is regulated by the 5′ AOX1 methanol-inducible promoter and secreted into the culture medium under direction of the α-factor signal sequence (SS). Asterisks represent the locations of mutations introduced to eliminate N-linked glycosylations (see **Table 1**) in *P. pastoris*. Lnk = linker; M = c-Myc tag; H = His6 tag. (**B**) LG-CBD accumulation in *P. pastoris* culture medium over time. A Coomassie-stained SDS−PAGE (upper image) and corresponding immunoblot (lower image) containing equivalent loadings of culture medium from 6 time points are shown. The immunoblot was probed with anti-His6 primary antibody. The predicted molecular weight of LG-CBD is 63.2 kDa. Molecular weight markers are given in kDa.

To confirm the binding of scFv to immobilized LG-CBD was specific, approximately 50 μg of anti-GBS scFv was passed over 1 mL of either CP-102 cellulose, protein L-agarose, protein G-agarose, or CP-102 + LG-CBD in a 10 mL BioRad column, washed, and eluted as indicated by the manufacturer's instructions or as noted above. Eluates were analyzed by SDS−PAGE.

Functional Characterization of Purified scFv Antibodies. To determine if purified anti-GBS scFv was capable of binding its target antigen, ELISA was performed essentially as described before (*27*). Briefly, 100 μg/mL of peptide−BSA conjugate diluted in bicarbonate buffer (0.15 M sodium carbonate and 0.35 M sodium bicarbonate, pH 9.6) was incubated (100 μL/well) for 16 h in 96-well plates (Nalgene-Nunc) at 4 °C and blocked with 200 μL of blocking buffer (2% (w/v) BSA prepared in PBS-T) for 1 h. Purified scFv (100 μL/well) was serially diluted from 50 μg/mL to 0.39 μg/mL in blocking buffer and incubated for 1 h. The plate was washed with PBS-T followed by incubation for 1 h with anti-His_6 IgG (Qiagen, Mississauga, Canada), diluted 1:5000 in blocking buffer. The plate was washed and incubated for 1 h with horseradish peroxidase (HRP) labeled goat antimouse IgG (MJS BioLynx; 100 μL/well), diluted 1:5000 in blocking buffer. After a final wash with PBS-T, TMB substrate (100 μL/well) was added, the reaction was stopped with 1.5 M H_2SO_4 (100 μL/well) and the absorbance was read at 450 nm on a Bio-Rad plate reader (Bio-Rad). Reaction controls included no coating antigen (i.e., no peptide-BSA), no scFv, or no anti-His_6 IgG.

RESULTS

Cloning, Expression and Purification of LG-CBD. To develop an affinity reagent with broad antibody-binding specificity that could be directly immobilized onto a cheap support matrix (cellulose) without the need for purification, we selected the previously characterized protein LG (*24*) and fused it to a family 2a cellulose-binding domain, creating LG-CBD (**Figure 1**). In synthesizing the DNA encoding LG-CBD, nucleotides were codon-optimized for eukaryotic expression and six putative

Table 2. Binding Capacity of Purified LG-CBD for Three Cellulose Resins

cellulose trade name	type	particle diameter (μm)	bulk density (g/cm^3)	mass (mg) of 1 mL of cellulose	binding capacity a	binding capacity b
Avicel PH-101	powder	~50	0.298	350	6.3 (±0.22)	34.8 (±1.21)
Celphere SCP-101	beads	~143	0.640	425	2.6 (±0.15)	17.4 (±1.01)
Celphere CP-102	beads	~160	0.870	650	2.5 (±0.18)	25.6 (±1.85)

[a] μg of LG-CBD mg^{-1} of cellulose dry weight; standard error of the mean (SEM). [b] nmol LG-CBD ml^{-1} of cellulose resin; (SEM).

N-glycosylation sites were removed. Samples of the *P. pastoris* culture medium revealed the accumulation of LG-CBD (63.2 kDa) over time (**Figure 1B**). We observed a higher molecular weight band on the Western blot which does not appear in subsequent SDS−PAGE gels (see below). One liter of culture yielded approximately 25 mg of LG-CBD after IMAC purification.

Binding Capacity of LG-CBD for Cellulose Resins. To determine the binding capacity of purified LG-CBD for various microcrystalline cellulose resins, a range of LG-CBD concentrations were incubated with a constant mass of cellulose. LG-CBD had the highest binding capacity for Avicel PH-101 (6.3 μg/mg cellulose), followed by Celphere SCP-101 (2.6 μg/mg cellulose) and Celphere CP-102 (2.5 μg/mg cellulose) (**Table 2**), which corresponds to a cellulose-binding capacity ranging from 34.8 to 17.4 nmol of LG-CBD per mL of cellulose resin.

Direct Immobilization of LG-CBD. To determine if LG-CBD expression could be interfaced with LG-CBD immobilization, microcrystalline cellulose resins were sterilized by autoclaving and added directly into cultures of *P. pastoris* expressing LG-CBD (**Figure 2**). Avicel PH-101, Celphere SCP-101, and Celphere CP-102 microcrystalline cellulose resins were evaluated as solid supports for direct LG-CBD immobilization in culture. Due to the low bulk densities of Avicel PH-101 and Celphere SCP-101 (**Table 2**), harvesting of treated resins from cell debris was impractical due to long settling times, resulting in contamination of the affinity resin with yeast cells. However, the high bulk density of Celphere CP-102 (**Table 2**) allowed easy harvesting, without yeast contamination, and as a result the resin was used for all further experiments involving direct LG-CBD immobilization. After harvest, LG-CBD was found to be immobilized onto the CP-102 resin with no contaminating proteins being detected by SDS−PAGE (**Figure 2**). Control experiments involving pPICZαA vector-only *P. pastoris* transformants and CP-102 indicated that no detectable proteins were immobilized on the CP-102 resin. Several preparations yielded a range of 0.77−1.54 μg of LG-CBD/mg of CP-102 beads. This yield was determined by densitometric analysis of SDS−PAGE gels containing BSA standards and serial dilutions of LG-CBD fusion protein removed from CP-102 resin (data not shown). These data were confirmed by elution of LG-CBD from CP-102 with 500 mM NaOH followed by absorbance measurements at A_{280}. These data illustrate a single-step method for the production of an immobilized affinity reagent by interfacing protein expression with solid-phase coupling *in vivo*.

Functional Characterization of the LG-CBD Antibody-Binding Domains. The anti-*Pseudomonas aerugonisa* IgG1 monoclonal antibody (*7*) or anti-group B *Streptococcus* (GBS) scFv antibody fragment (*27*) were passed over immobilized LG-CBD or control purification resins (CP-102, protein L-agarose, protein G-agarose) to determine LG-CBD functionality. As shown by SDS−PAGE analysis of the eluates (**Figure 3**), IgG1 bound to protein G-agarose and LG-CBD. The IgG did not bind to CP-102 cellulose resin or protein L-agarose as expected. The anti-GBS scFv bound and was eluted from protein L-agarose and LG-CBD, but not CP-102 cellulose resin alone or protein G-agarose as expected since this scFv contained a Vκ1 light chain. These results

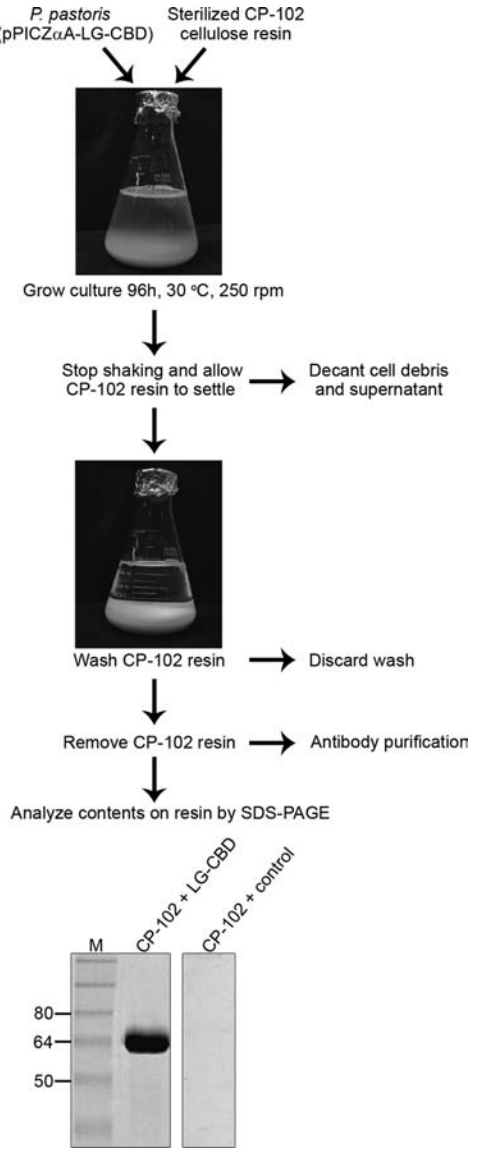

Figure 2. Expression of LG-CBD and direct immobilization onto microcrystalline cellulose resin. *P. pastoris* cells expressing LG-CBD were grown in the presence of CP-102 microcrystalline cellulose resin (top). Particles were allowed to settle by gravity after 96 h (middle) and then washed; any protein bound to the resin was eluted and analyzed by SDS−PAGE (bottom left image). In control experiments involving *P. pastoris* cells transformed with pPICZαA empty vector (control), no detectable proteins are bound to CP-102 resin (bottom right image). Molecular weight markers are given in kDa.

indicated both the protein L and protein G domains of LG-CBD were functional and antibody-binding was not significantly hindered by fusion of these domains to the CBD.

Purification of IgG1 from Tobacco and Activity Profiling. To determine if immobilized LG-CBD could be used as a purification tool for plant-expressed antibodies, transgenic tobacco

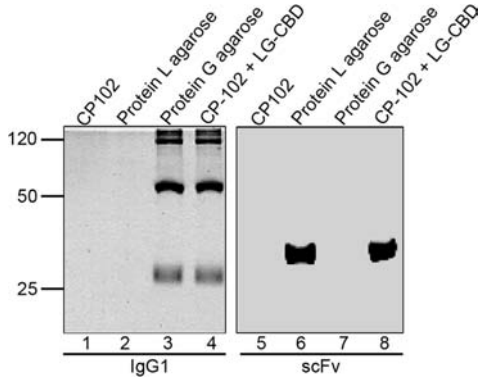

Figure 3. Protein L and protein G domains of LG-CBD are functional when immobilized on microcrystalline cellulose. The antibody binding properties of immobilized LG-CBD components are shown. Eluted proteins from each resin were analyzed by SDS−PAGE. Protein G-agarose and CP-102 + LG-CBD were capable of binding a κ2 light chain-containing IgG1 (left image), whereas protein L-agarose and CP-102 + LG-CBD were capable of binding a Vκ1-containing scFv (right image). Molecular weight markers are given in kDa on the left, and purification resins used are given along the top.

producing the aforementioned anti-*Pseudomonas* IgG1 was grown as a test model (*7*). CP-102 resin saturated with LG-CBD was batch incubated with homogenized wild-type (control) or IgG1-expressing tobacco leaves, washed, and IgG selectively eluted using a low pH glycine buffer. The IgG1 was purified to near homogeneity by a series of elutions, as illustrated by SDS−PAGE analysis under reducing conditions (**Figure 4A**). To confirm the eluted protein was the anti-*Pseudomonas* IgG1, concentrated and dialyzed eluates were subjected to immunoblot analysis under nonreducing and reducing conditions and probed with either heavy-chain specific (α-His6) or Fab-specific antibodies (**Figure 4B**). Approximately 0.4 mg (±0.08) of IgG1 was purified from 125 g of tobacco, corresponding to a yield of 3.2 mg of IgG1 per kg of tobacco. We have previously estimated the level of IgG expression in this tobacco line at 4−5 mg of IgG per kg of biomass by semiquantitative Western blotting (ref *20*, and data not shown). Our yield therefore corresponds to a recovery of 64−80% of total IgG. Binding of anti- *Pseudomonas* IgG1 to immobilized LG-CBD was facilitated through the protein G subunit and not influenced by protein L or passive adsorption to CP-102, as demonstrated (**Figure 3**). ELISA illustrated that anti-*Pseudomonas* IgG1 purified by this method retained its ability to bind target antigen: either heat-killed *P. aeuroginosa* O6ad or LPS derived from the same strain (**Figure 4C**). Furthermore, when IgG1 purified by LG-CBD was compared to the same IgG1 molecule purified by the two-step protocol of McLean et al. (*7*) , no significant differences were found in the binding of IgG1 to O6ad when equivalent concentrations of IgG1 where compared by ELISA (**Figure 4D**). These results suggest that immobilized LG-CBD is capable of purifying IgG1 from transgenic tobacco to comparable purity achieved by the two-step method of McLean et al. (*7*) , based on direct comparison by ELISA.

Coexpression, Purification and Activity Profiling of a scFv Antibody Fragment. One of the major objectives of this paper is the coexpression of protein-based immunosorbents (e.g., CBD-LG on cellulose beads) and the antibody-based target of interest (e.g., IgG, scFv) for concurrent isolation of the antibody target on the immunosorbent in a process we refer to as "co-expression affinity purification". As an initial proof of concept, we grew two *P. pastoris* strains together, (i.e., one capable of secreted expression of LG-CBD and the other capable of secreted scFv expression), all in the presence of CP-102 resin. LG-CBD was capable of binding both CP-102 cellulose and scFv in culture (**Figure 5A**). Furthermore, scFv could be selectively eluted from LG-CBD using a low pH glycine eluent (**Figure 5A**, lanes 6−9). Densitometric analysis of proteins stripped from CP-102 cellulose resin before (**Figure 5A**, lane 5) and after (**Figure 5A**, lane 10) scFv elution revealed 84.6% of LG-CBD and 22.3% of scFv remained on the resin after elution. This indicates nearly 80% of the scFv could be selectively eluted from the affinity resin while only a small fraction of LG-CBD was removed. The anti-GBS scFv did not passively adsorb to CP-102 resin (**Figure 5A**, lane 3; **Figure 3**, lane 5), and binding to LG-CBD was facilitated by the protein L domain and not protein G, as shown (**Figure 3**). The scFv expression level was estimated at 5 mg/250 mL (data not shown). From our coexpression system we were capable of eluting approximately 1.75 mg of scFv from immobilized LG-CBD, which corresponds to a recovery of 35%.

In addition, ELISA was used to illustrate that purified anti-GBS scFv was capable of binding its target antigen, a BSA-peptide mimitope (**Figure 5B**). Taken together, we provide a model which simulates the simultaneous production of target protein (i.e., antibody) and capture reagent within the same organism for rapid purification of functional antibodies.

DISCUSSION

Commercial downstream processing and purification of pharmaceutical proteins from traditional sources (i.e., CHO cells, *E. coli*) accounts for 50−80% of total manufacturing costs (*28*), and is one of the major obstacles to the production of pharmaceutical proteins from nontraditional sources such as crop plants. Most biological affinity ligands currently in use for antibody purification require expensive and laborious production, purification, and coupling steps before use (*29*). To explore alternative purification schemes, we have developed a simple method for immobilizing CBD-tagged affinity ligands onto solid supports and demonstrated its utility by purifying both monoclonal antibodies and antibody fragments. We reasoned that by simplifying the coupling process of affinity ligands to inexpensive and inert cellulose matrices, while maintaining immobilization densities and antibody-binding capacities of commercial reagents, one could reduce downstream processing costs.

With this objective in mind, we expressed a trifunctional fusion protein in yeast consisting of the antibody-binding domains protein L and protein G fused to a CBD (**Figure 1A**). We chose to use both affinity domains due to the broad range of Abs and Ab fragments that will bind to them (*24*, *30*). We chose to use a family 2a CBD for immobilization of the affinity domains because of its high-affinity and nearly irreversible binding to microcrystalline cellulose (*19*), amenability to production in *P. pastoris* (*25*), and negligible impact on the function of the fusion partners (*12*, *14*). We codon-optimized the amino acid coding sequences for the entire LG-CBD fusion protein to increase recombinant protein expression (*31*) and 6 amino acid residues were mutated (**Table 1**) to prevent N-linked glycosylation by the expression host *P. pastoris*. SDS−PAGE analysis of LG-CBD revealed a single band at the expected molecular weight of 63.2 kDa, however, Western blot analysis of *P. pastoris* supernatants containing LG-CBD revealed a second higher molecular weight band (**Figure 1B**). The higher molecular weight band is not be visible by less-sensitive SDS−PAGE staining and may correspond to any of the following: (i) incomplete cleavage of the α-factor secretion peptide from LG-CBD, (ii) partial O-linked glycosylation of LG-CBD, or (iii) N-linked or O-linked glycosylation of the incompletely cleaved α-factor secretion peptide from LG-CBD. Since LG-CBD retained its functionality, we did not

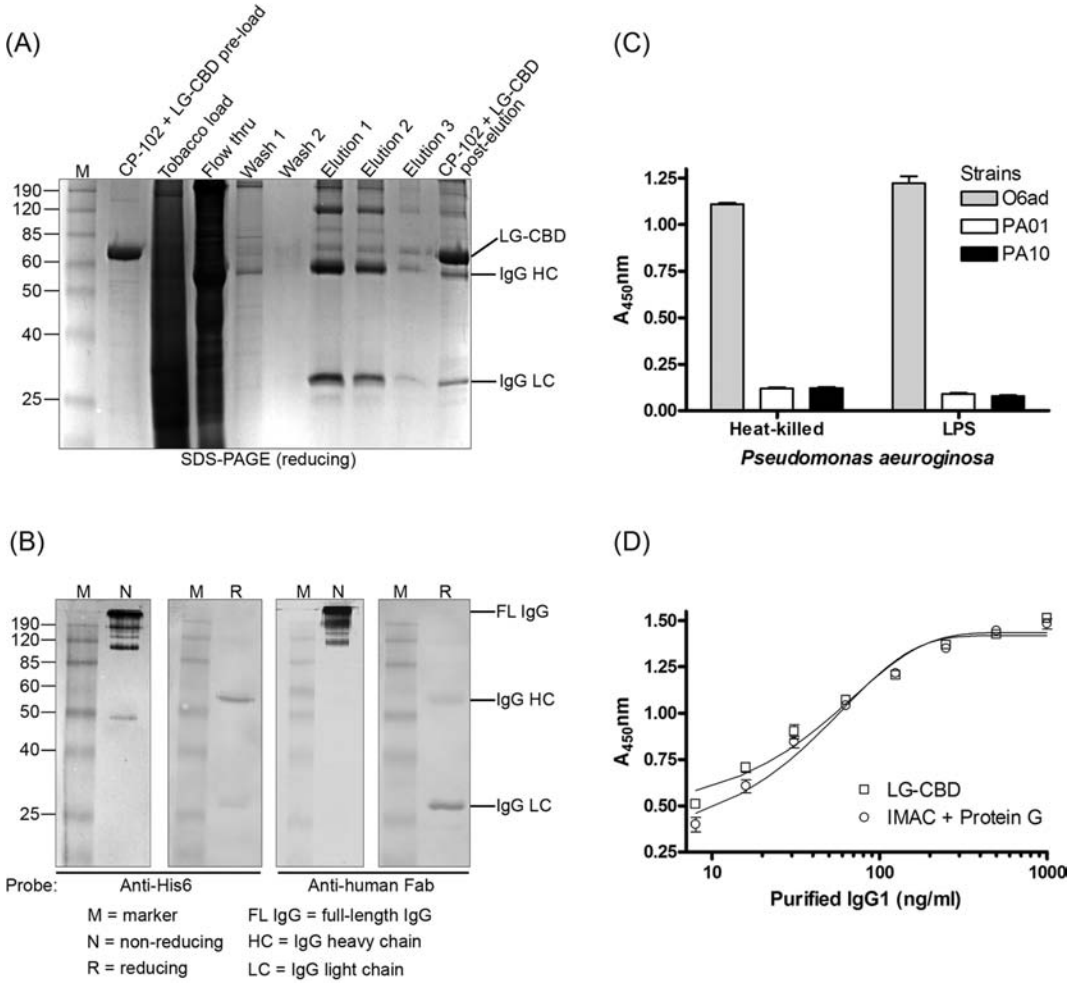

Figure 4. Purification and activity profiling of a human IgG1 mAb from tobacco. (**A**) LG-CBD immobilized on CP-102 was capable of purifying a human IgG1 mAb expressed in tobacco. A reducing SDS−PAGE is shown, containing LG-CBD before application of feedstock (CP-102 + LG-CBD preload), primary feedstock (tobacco load), flow through and 2 washes. The mAb was selectively eluted from CP-102 + LG-CBD resin (elutions 1, 2 and 3) with only minor amounts of mAb remaining on the affinity resin (CP-102 + LG-CBD postelution). The positions of LG-CBD and IgG heavy (HC) and light (LC) chains are indicated on the right. Molecular weight markers are given in kDa on the left of both (**A**) and (**B**). Immunoblot (**B**) and ELISA (**C**) analyses confirm the identity and functionality of the purified mAb. (**B**) Reducing and nonreducing immunoblot analyses. Two immunoblots on the left were probed with anti-His6 primary antibody; two immunoblots on the right, with antihuman Fab (which selectively binds light chains). Letter key is given at the bottom. (**C**) ELISA showing that CP-102 + LG-CBD purified antibody binds specific antigenic targets. Specific (O6ad) and nonspecific (PA01 and PA10) bacterial strains used as coating agents, and their LPS, are indicated in the upper right. (**D**) ELISA with IgG1 purified by either a single-step method (LG-CBD) or two-step method (IMAC + protein G) illustrating similar IgG1 activity for *P. aeuroginosa* strain O6ad coating agent. Key: squares = LG-CBD purified antibody; circles = IMAC followed by protein G purified antibody; IMAC = immobilized metal-affinity chromatography.

explore the nature of the doublet any further. We chose *P. pastoris* strain X-33 as a host because of its ability to secrete recombinant proteins containing the *Saccharmoyces cerevisiae* α-factor signal sequence, its characteristic of secreting few native proteins, and its potential for large-scale growth in fermentation bioreactors (*32*).

The immobilization of CBD-tagged fusion proteins generally involves expression and binding to a solid cellulose support matrix. We chose to add different cellulose resins directly to *P. pastoris* cultures expressing LG-CBD. Two of the resins tested, Avicel PH-101 and Celphere SCP-101, were difficult to separate from the *P. pastoris* cells and debris because of their low bulk densities (i.e., 0.298 g/cm^3 and 0.640 g/cm^3, respectively). Celphere CP-102 had a higher bulk density (i.e., 0.870 g/cm^3) and was easily separated from culture debris by decanting the *P. pastoris* cells and washing the resin. As a result, we could immobilize up to 1.54 μg of LG-CBD per mg of CP-102 resin and determined the resin to be free of nonspecifically bound proteins. Compared to commercial affinity resins such as protein A-agarose or protein G-agarose which require purified ligands and chemical cross-linking steps (*33*), this direct immobilization method reduces the time required to prepare such affinity reagents.

Using immobilized LG-CBD, we were able to recover ∼3.2 mg of IgG/kg of biomass, or ∼80% of the total IgG1 recovered using commercially available reagents (IMAC + Protein G) (*7*). Through semiquantitative Western blot analysis (data not shown) we estimated the IgG expression level at 4 - 5 mg of IgG per kg of tobacco, giving our system a percent recovery of 64 - 80%. We determined that binding of anti-*Pseudomonas* IgG1 to LG-CBD was only mediated through the protein G subunit and not influenced by protein L or the CP-102 support matrix. This was expected since human IgG1 contains a Vκ2 light chain, which, in addition to λ light chain-containing Abs, is not recognized by protein L (*29*). Furthermore, very little IgG remained on the LG-CBD cellulose resin after elution indicating near-complete dissociation of Ab and ligand upon exposure to eluent. We then showed, by ELISA, that purified IgG1 retained its antigen-binding specificity for *P. aeuroginosa* strain O6ad. Finally, we used ELISA to compare equivalent concentrations of

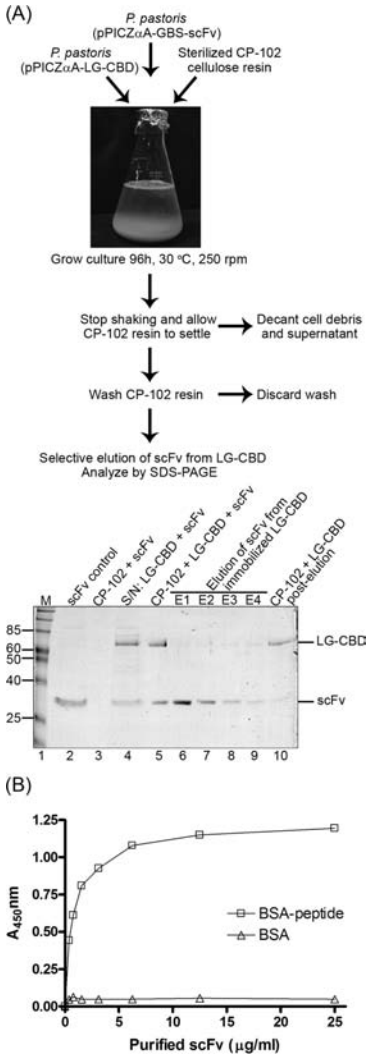

Figure 5. Coexpression and cocapture of affinity ligand (LG-CBD) and antibody (scFv) on microcrystalline cellulose. (**A**) Coexpression of LG-CBD and anti-GBS scFv by 2 *P. pastoris* strains in the presence of CP-102 cellulose resin within a single culture allows simultaneous binding of LG-CBD affinity ligand to both cellulose resin and antibody. Procedural schematic is shown below the top image. The scFv could be selectively eluted from CP-102 + LG-CBD, as shown in the SDS−PAGE (bottom image). Lane 1: molecular weight markers, given on the left in kDa. Lane 2: supernatant from a culture expressing scFv only (control). Lane 3: elution by boiling profile from CP-102 resin added to scFv-only expressing culture, indicating that scFv does not bind to CP-102 cellulose (control). Lane 4, sample of 96 h supernatant (S/N) from a culture containing two *P. pastoris* strains expressing scFv and LG-CBD (control). Lane 5: elution by boiling profile from CP-102 resin added to a culture containing two *P. pastoris* strains after expression of scFv and LG-CBD, indicating that CP-102 resin binds LG-CBD and associated scFv. Lanes 6−9: scFv elutions using low pH glycine (E1−4) from CP-102 resin present in a culture during expression of both scFv and LG-CBD (as in top image). Lane 10: elution by boiling profile (i.e., resin stripping profile) from CP-102 resin in lane 9 post elution. The positions of LG-CBD and the scFv are indicated on the right. (**B**) ELISA confirming the eluted scFv is functional and can bind specific peptide antigen conjugated to BSA. Squares = specific peptide epitope conujugated to BSA; triangles = BSA control. The amount of purified scFv used per treatment is indicated along the bottom; absorbances at 450 nm are at the left.

IgG1 purified by either the one-step LG-CBD method or by the method of McLean et al. (*7*), and found no significant differences in specific antigen binding activity. Therefore, when compared to the method of McLean et al. (*7*), functional IgG1 could be purified in fewer steps from transgenic plants using LG-CBD immobilized on cellulose resin. While we acknowledge that we did not perform a comprehensive comparison to commercial affinity resins with respect to antibody-binding capacity, recovery yields, lifespan, and cost, our aim here was to simply explore the possibility of directly immobilizing a CBD-tagged fusion protein onto cellulose and demonstrating its functionality thereafter.

Werner et al. (*34*) have suggested that an ideal purification reagent for plant-based pharmaceuticals is a low-cost, single-use unit. *In planta* fusions of therapeutic proteins to intein (*35*), integral membrane-spanning domains (*36*), or GUS (*37*), and fusion of seed-expressed Abs to oilbodies (oleosin-fusion technology) (*38*) are all promising purification strategies, although most require cleavage of the fusion partner. More recently, *in planta* display of two subunits of protein A nanoparticles by tobamovirus (*34*) was shown to efficiently capture plant-expressed mAbs. Similar to the work of these researchers, we were able to coexpress an immunosorbent (LG-CBD) with an Ab (anti-GBS scFv) in the same culture containing a solid support matrix (cellulose) and found LG-CBD immobilized and associated with scFv. As expected, the protein L subunit of LG-CBD was solely responsible for anti-GBS scFv binding since this scFv contains a VκI light chain and lacks an Fc region, the recognition domain of protein G (*29*). Furthermore, functional scFv could be selectively eluted from the immobilized LG-CBD affinity ligand.

Although the potential for binding of CBD-tagged proteins to plant structural cellulose must be further investigated, our protocol for purification of IgG antibody from tobacco exposes LG-CBD already immobilized on microcrystalline cellulose beads to tobacco leaf homogenates that have been extensively clarified of insoluble cellulose material. As such, we have no reason to believe that LG-CBD would have any plant cell cellulose to bind to. Furthermore, the binding of the CBD for microcrystalline cellulose is considered nearly irreversible ($K_a = 10^6-10^7$ M^{-1}): virtually zero CBD desorption was observed upon disturbing the equilibrium in CBD binding experiments performed by Tomme et al (*9*). If there was any insoluble plant cell wall cellulose present in our batch purification mixture, only a small fraction of LG-CBD would dissociate from the cellulose beads and associate with it, maintaining an efficiency of target molecule purification that should be acceptable with large-scale processes.

In conclusion, this work demonstrated a method by which a functional affinity ligand can be expressed and directly coupled onto a solid support matrix in real time during the growth of the expression host. As a proof of concept, this work also showed that a single yeast culture can simultaneously produce both a bioproduct of interest (antibody) and a functional affinity ligand (LG-CBD). Straightforward preparation of purification reagents like this may help make the efficient and inexpensive purification of recombinant antibodies from crop plants feasible. Furthermore, it would be interesting to determine if antibodies and CBD-tagged affinity ligands expressed within the same plant could coassociate on insoluble cellulose and be exploited for antibody recovery at commercial scales.

ABBREVIATIONS USED

Ab, antibody; BSA, bovine serum albumin; CBD, cellulose-binding domain; CHO, Chinese hamster ovary; EBA-IMAC, expanded-bed adsorption-immobilized metal affinity chromatography; ELISA, enzyme-linked immunosorbent assay; GBS, group B *Streptococcus;* HRP, horseradish peroxidase; IgG, immunoglobulin G; IMAC, immobilized metal affinity chromatography;

kDa, kilodalton; LG-CBD, protein L/protein G/cellulose binding domain fusion protein; mAb, monoclonal antibody; PBS, phosphate buffered saline; PBS-T, PBS Tween 20; scFv, single chain variable fragment; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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