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Construction of pRSET-*sf*GFP Plasmid for Fusion-Protein Expression, Purification and Detection

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Abstract

Green fluorescent protein (GFP) is widely used as an excellent expression tag for fusion proteins, which can improve their expression while preserving their function and native-like structure. Fusion protein method allows the purification and the detection of a protein of interest even when no specific antibody is available. This study aimed to design a system for protein expression and detection using a new super-folder derivative of GFP (*sf*GFP) as a fusion partner. This included the construction of the protein expression plasmid pRSET-*sf*GFP by cloning *sf*GFP gene downstream of the N-terminal 6×His tag in the T7 promoter-plasmid pRSET. The soluble expressed *sf*GFP protein (27 kDa) from this plasmid in the cytoplasm of *E. coli* was purified using metal affinity chromatography, as shown after SDS-PAGE separation and blue gel staining. In order to detect *sf*GFP, as single or in fusion proteins, in ELISA and immunoblotting analysis, *sf*GFP-specific polyclonal antibodies were produced in rabbit after immunization with three injections with the pure *sf*GFP prepared in Freund's adjuvant. Two-step antibody purification, using protein A-Sepharose and *sf*GFP-coupled Sepharose affinity chromatography columns, was performed to obtain highly reactive and pure *sf*GFP-specific IgG. This system will provide an efficient tool for the expression, purification and detection of many proteins, having problems in solubility and stability, as fusion partners with *sf*GFP.

Keywords: *sf*GFP, Gene cloning, protein expression, polyclonal antibody.

1. Introduction

The green fluorescent protein (GFP) from *Aequorea* jellyfish has become a common label for many *in vivo* and *in vitro* applications due to GFP's ability to fold and form a visually fluorescent chromophore through autocatalytic cyclization and dehydration/oxidation reactions (Shimomura *et al.*, 1962). GFP is exceptionally stable in solution, even at high temperature, in the presence of organic solvents or alkaline pH condition. Three dimensional X-ray crystallography reveals that GFP is tightly folded in a form of a barrel of β sheets, resulting in a stable protein structure (Ormo *et al.*, 1996). For example, high concentration of chaotropic reagents is able to unfold many proteins, but GFP does not lose its fluorescence even in 6M-urea solution. In addition, fluorophore of GFP is insensitive to the low temperature, so freezing at -20 degree for long-term storage does not affect its green fluorescence (Puckett *et al.*, 2004). GFP fluorescence is very stable and does not require any substrate or cofactor (Inouye and Tsuji, 1994). It absorbs blue light (absorption wavelength peak, 395 nm) and emits green light (emission wavelength peak, 508 nm) that is detectable using a fluorescence microscope or a fluorescence-activated cell sorter (FACS) (Limon *et al.*, 1997). Because of the interesting spectral and structural features of GFP, it has been widely used as a reporter molecule in several studies of protein folding (Waldo *et al.*, 1999; Waldo, 2003), protein-protein interactions (Southward and Surette, 2002; Wilson *et al.*, 2004; Cabantous *et al.*, 2005a; Cabantous *et al.*, 2005b; Magliery *et al.*, 2005) and gene translation (Subramanian and Srienc, 1996; Kalir *et al.*, 2001; Blokpoel *et al.*, 2003).

GFP is a 238-amino acid polypeptide with a molecular weight of 27 kDa and its encoding cDNA was cloned and sequenced in 1992 (Prasher *et al.*, 1992), opening the way for expression of the protein in heterologous systems. GFP has been expressed in a variety of species, including bacteria, plants, *Drosophila melanogaster*, *Caenorhabditis elegans*, zebra fish, and mammals (Chalfie *et al.*, 1994; Gerdes and Kaether, 1996). Recently, two studies reported the creation of GFP superproteins, which are high performance proteins optimized for *in vivo* functions

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(Lansbury, 2001; Vendruscolo and Dobson, 2007). Furthermore, Waldo and coworkers reported the engineering of a superfolder GFP (*sf*GFP) that showed improved tolerance to circular permutation, increased resistance to denaturation, improved folding kinetics, and increased resistance to aggregation during refolding (Pedelacq *et al.*, 2006; Andrews *et al*., 2007; Fisher and DeLisa, 2008). *sf*GFP has proven to be very useful as a scaffold for improved protein detection and tagging both *in vivo* and *in vitro* using self-assembled *sf*GFP fragments (Cabantous *et al.*, 2005b; Cabantous and Waldo, 2006). Furthermore, *sf*GFP fusions are more soluble than conventional GFP fusions (Wu *et al.*, 2009). Many kinds of GFP-fused proteins have been produced and studied in cell biology. Such fusion does not necessarily alter the main function and structure of the protein of interest, e.g. a *Drosophila* protein was fused to GFP to visualize its distribution in the living cells (Wang and Hazelrigg, 1994).

For efficient purification, 6×His tag was added to the N-terminal of GFP in some approaches (Kim *et al.*, 2007; Chiang *et al.*, 2008; Lee, 2009; Liu and Naismith, 2009). Due to its small size (~2 kDa), 6×His tag fusion does not make a problem related to larger molecular weight of fusion proteins. Since GFP does not have an affinity to divalent cation, binding and elution of 6×His tag is unlikely to inhibit the function of the fusion protein. In addition, the pH range of 6×His tag purification process is generally kept between 7 to 8, which is optimal for green fluorescence of GFP. Although, high salt condition of buffers or the presence of imidazole is not problematic to the stability or fluorescence of GFP (Lee, 2009).

Antibodies are an important tool used by many investigators in the research and have led to many medical advances. In biochemical and biological researches, polyclonal antibodies are routinely used as ligands for the preparation of immunoaffinity columns (Shin *et al.*, 2001) and as coating or labeling reagents for the qualitative and quantitative determination of molecules in a variety of assays, such as enzyme linked immunosorbent assay (ELISA) and immunoplotting (Calabozo *et al.*, 2001; Cheung *et al*., 2002).

This study aimed to design a system for *sf*GFP-fused protein expression and purification using pRSET plasmid, which allows high level of expression, due to its T7 promoter, of N-terminal 6xHis tagged recombinant proteins in *E. coli*. After purification with Nickel affinity chromatography, pure *sf*GFP could be used to generate and select specific rabbit polyclonal antibody. Anti-*sf*GFP antibody is of a great utility for the detection of GFP, as well as GFP-fused proteins, in ELISA and immunoblotting applications, following protein expression and purification. Such protein fusion system should provide an efficient tool for high yield expression and fast detection for many proteins having problems in stability and solubility.

2. Materials and Methods

2.1. . Bacterial strains, growth conditions and plasmid

E. coli strains TOP10 (Invitrogen) and BL21 (DE3) Rosetta (Novagen) were used in cloning and protein expression after transformation by electroporation with the plasmid pRSET (Invitrogen). For general maintenance and protein expression, *E. coli* were grown in Luria Broth (LB; 1% Tryptone, 0.5% yeast extract, 171 mM NaCl) (Bio Basic INC) with ampicillin antibiotic (Sigma; 100 μg/ml) in orbit-rotating 37°C incubator.

2.2. Plasmid construction

The open reading frame of *sf*GFP gene was amplified by a high fidelity PCR (AccuPrim™Taq Polymerase High fidelity Kit; Invitrogen) from a pT7-His plasmid (kindly provided by Prof. Yu Ding, Fudan University, China) using a pair of specific primers: *sf*GFP F-*Nhe*I (5'-ATATAT**GCTAGC**ATGAGCAAAGGCGAAGAACT-3') and *sf*GFP R-*Bam*HI (5'-ATATAT**GGATCC**GCCATGAGTGATACCCGCCG-3'). Primers were designed to amplify the full length of the gene without the start and stop codons, and to add *Nhe*I and *Bam*HI (Fermentas) restriction sites at the 5' and 3' ends, respectively. PCR amplification high fidelity program consisted of 3 min of denaturation step at 94 °C followed by 35 cycles of short denaturation step at 94 °C for 30 sec, annealing at 55°C for 30 sec and extension at 72 °C for 45 sec. Amplified *sf*GFP fragment and pRSET plasmid were digested with *Nhe*I and *Bam*HI and then ligated using T4 DNA ligase (Fermentas). Freshly prepared electro-competent *E. coli* TOP10 cells were transformed with the new plasmid construct pRSET-*sf*GFP by electroporation. Colony PCR screening for positive *sf*GFP clones was performed using pRSET specific primers (TF/TR). Plasmid constructs were isolated from some positive clones by Plasmid Miniprep Kit (Qiagen) after being grown in LB/ampicillin medium. Successful cloning of these plasmid constructs was confirmed by digestion with restriction enzymes and by sequencing.

2.3. Expression and purification of soluble sfGFP protein In E. coli

Confirmed plasmid construct was used to transform by electroporation *E. coli* BL21 (DE3) Rosetta cells. Protein expression of *sf*GFP was performed in 250 ml shake flasks by growing the bacteria in LB medium till an optical density of 0.5 to 0.7 was reached and then expression was induced with 0.5 mM IPTG (Isopropyl β-D-thiogaldctoside; Promega) for 16 h at 19 °C. After pelleting the cells, the pellet was resuspended in PBS 1X, then lysed by sonication (Lab Sonic) on ice and the lysate was cleared by 20 min centrifugation at 8,000 rpm and 4 °C. Using fast protein liquid chromatography (FPLC) AKTA prime plus system (GE life science), recombinant *sf*GFP was purified from the cytoplasmic extract using a 5 ml column of Nickel charged Nitrilotriacetic acid (NTA) superflow Sepharose (Qiagen). After washing, the bound proteins were eluted from the column with a 500 mM Imidazole buffer. The eluted fraction was concentrated on Vivaspin concentrators with a molecular mass cutoff of 10 kDa (Vivascience). The concentration of the purified protein was determined by Bradford method. The purity of the *sf*GFP was evaluated in a Coomassie-stained SDS-PAGE. Protein samples were separated by SDS-PAGE using a Bio-Rad mini-Protean II system following the manufacturer's instructions. Gels were prepared using stacking gel 5 % and running gel 12 %. After electrophoresis, the gel was stained in Coomassie brilliant blue buffer (45 % Methanol, 10 % Acetic acid, 0.25%

Coomassie R250) for 2 h and then washed several times in destaining buffer.

2.4. Production of polyclonal antibody against the recombinant sfGFP Protein

For the first immunization, purified *sf*GFP protein (500 µg) in PBS (1 ml) was mixed with an equal volume of Freund's complete adjuvant (Bio Basic Inc.) to form a stable emulsion. One white rabbit was injected subcutaneously at 2 to 4 different sites. Three booster injections were given with 250 µg recombinant protein mixed with incomplete Freund's adjuvant at 15 days intervals. To evaluate the immune response, blood samples (0.5-1 ml) were collected before each injection and bleeding (30 ml) was done 10 days after the last boost. Polyclonal antibodies (IgGs) were purified from rabbit serum by affinity chromatography on a 5 ml HiTrap Protein A column (GE life science) according to the manufacturer's instructions. Binding was performed in 0.02 M sodium phosphate, pH 7 and rabbit IgGs were eluted with 0.1 M Citric acid, pH 3. Eluted IgGs were collected and immediately neutralized to physiological pH with 1 M Tris-base buffer, pH 9 and then concentrated to 1mg/ml on Vivaspin concentrators with a molecular mass cutoff of 50 kDa (Vivascience).

2.5. Affinity purification of sfGFP IgG

NHS-activated Sepharose Column (GE lifescience) was used for affinity purification of *sf*GFP-specific IgGs from the rabbit total antibodies, which were obtained in the previous step. Before immobilization on NHS-activated column, dissolving buffer of *sf*GFP was exchanged with the standard coupling buffer (0.2 M $NaHCO_3$, 0.5 M NaCl, pH 8.3) using HiPrep™ 26/10 Desalting Column (GE life science). Concentrated *sf*GFP (20 mg) was applied onto NHS-activated column (1 ml) and allowed to bind for 30 min at 20°C. Any excess of active NHS (N-hydroxysiccinimide) groups that have not coupled were deactivated by sequential washing with (0.5 M Ethanolamine, 0.5 M NaCl, pH 8.3) and (0.1 M Sodium Acetate, 0.5 M NaCl, pH 4.0). Protein-A purified IgGs from rabbit serum were passed through the *sf*GFP-column. Unbound IgGs were washed away with the washing buffer (0.05 M Sodium Phosphate, 0.15 M NaCl, pH 7.0) and bound IgGs were eluted with 3 – 5 column volumes of 0.1 M Citric acid buffer and then neutralized with 1 M Tris-base buffer, pH 9. Purified *sf*GFP-specific IgG sample was buffer exchanged with 0.05 M Sodium phosphate buffer pH 7, and then concentrated down to 1 mg/ml on Vivaspin concentrators with a molecular mass cutoff of 50 kDa.

2.6. ELISA assay for the detection of anti- sfGFP

An indirect ELISA format was employed for the analysis of sera. The basic protocol was as follows: *sf*GFP antigen was diluted in coating buffer (0.2 M sodium acetate, pH 5) and added to the wells (100 µl) of ELISA plates at 4 °C overnight. After coating, ELISA plate was washed 5 times with washing buffer (1 × PBS containing 0.05 % Tween-20) to remove unbound antigens, 200 µl of blocking buffer (1% bovine serum albumin (BSA), 3 % skimmed milk in 1 × PBS) was applied to all wells for two hours at 37 °C. After the removal of blocking buffer, rabbit sera or the commercial mouse anti GFP (Roche), diluted in 1 % blocking buffer, were added per well (100 µl) for 1 h at RT. After 5 washes, goat anti-rabbit or anti-mouse IgGs (Bethyl laboratories) conjugated to horse-radish peroxidase (HRP) were diluted (1:4000) in 1% blocking buffer, added to the wells (100 µl), and allowed to bind to captured IgG for a further hour at RT. After an additional 5 washes, bound conjugate was detected with 50 µl of (3,3′,5,5′-Tetramethylbenzidine) TMB substrate (Sigma), the reaction was stopped after 8 minutes with the addition of 50 µl 1 M H_2SO_4. The spectroscopic absorbance of each well was measured in an automated plate reader at a wavelength of 450 nm.

2.7. Immunoblotting of sfGFP

SDS-PAGE (10 %) separation was performed to 0.2 µg of pure *sf*GFP and two recombinant *sf*GFP-fused proteins (homemade); with growth hormone (*sf*GFP-GH) and insulin-like growth factor (*sf*GFP-IGF). Separated proteins were blotted onto two 0.45 µm nitro-cellulose membranes (BioRad) using 1× blotting buffer (25 mM Tris-base, 200 mM Glycine, 0.1 % SDS and 20 % Methanol). After incubation in the blocking buffer (3 % skimmed milk, 1% BSA in 1 × PBS), one of the membranes was incubated with 1:2000 dilution of commercial mouse anti-GFP antibody and the other with pure rabbit anti-*sf*GFP IgG for an hour at RT. After several washes with TBS-tween20 (20 mM Tris-base, 150 mM NaCl, 0.05 % Tween-20, pH 7.5,), they were incubated with 1:2000 dilution of secondary goat anti-mouse or anti-rabbit antibodies, conjugated to alkaline phosphatase (AP) (Bethyl laboratories) for 1 h at RT. Bands revelations was achieved by adding Nitro blue tetrazolium chloride (NBT, 0.05 %)/5-bromo-4-chloro-3-indolyl phosphate (BCIP, 0.025 %) (Sigma) chromogen substrate in AP buffer (100 mM tris-base, 100 mM NaCl, 5 mM $MgCl_2$, pH 9.5).

3. Results

3.1. Cloning of sfGFP gene into pRSET plasmid

Plasmids with T7 promoter technology is widely used for high throughput protein expression in *E. coli* strains carrying the lambda DE3 lysogen (Studier and Moffatt, 1986; Studier *et al*., 1990). The plasmid pRSET is a T7 promoter-dependent system which produces cytoplasmic N-terminal 6×His-tagged proteins in *E. coli* cells (Fig. 1A). Tagging of recombinant proteins is indispensable for the subsequent steps of purification and detection. The full length gene of *sf*GFP was amplified from the plasmid pT_7-His which was used as a template in PCR amplification by two specific primers, resulting in the amplification of a single DNA fragment of 740 bp. Digestion of this DNA fragment resulted in a sticky ended fragment ready for ligation in linearized pRSET plasmid with the two restriction enzymes *Nhe*I, *Bam*HI (Fig. 1B). Ligated products were used to transform *E. coli* TOP10 cells by electric shock and positive colonies on the selective plates were screened by PCR using pRSET-specific primers (TF/TR). This approach enabled distinguishing between two types of colonies; empty pRSET-containing colonies, which resulted in small amplified DNA fragment (295 bp) and pRSET-*sf*GFP-containing colonies which gave a big fragment of 931 bp due to the presence of the insert gene within (Fig. 1C). Positive colonies were grown and used for plasmid mini-preparation and the correct structure of the plasmid constructs were verified by sequencing.

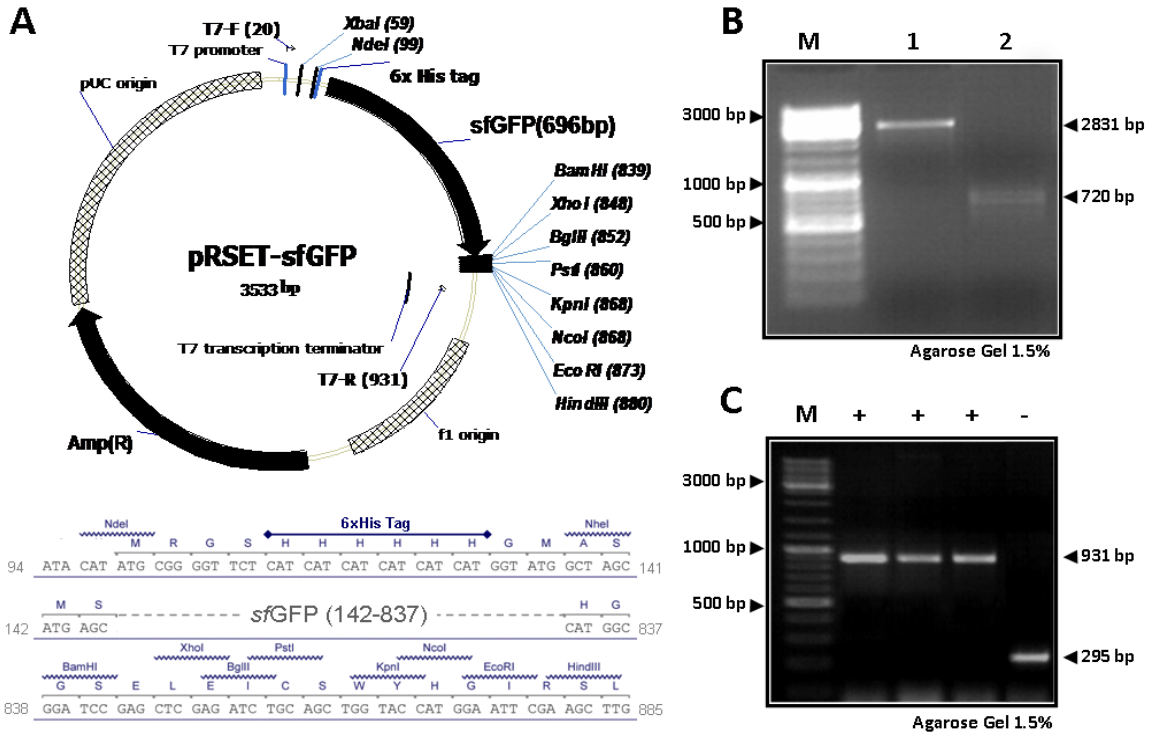

Figure 1. Cloning of *sf*GFP into pRSET plasmid
(**A**). Map of the resulted plasmid construct (pRSET-*sf*GFP) where inserted *sf*GFP gene is indicated. The most important elements of the plasmid are indicated, including T7 promoter, 6×His tag upstream the restriction sites for *Nhe*I and *BamH*I used for *sf*GFP gene ligation. Position of the two primers TR/TF used for PCR positive-colonies screening is indicated as well. Nucleotide sequence of the plasmid region between *Nde*I and *Hind*III (position from 94 to 885) and their predicted amino acid symbols according to the reading frame codons, is shown below. This region contains: 6×His tag (112-129), *sf*GFP gene (142-837) and its downstream multi cloning site (MCS) (838-885) for 8 different unique-cutting restriction enzymes. (**B**) DNA bands of pRSET plasmid (lane 1) and *sf*GFP PCR amplified gene (lane 2) after being digested with *Nhe*I/*BamH*II restriction enzymes before the ligation reaction. (**C**) Results of colony PCR screening performed on 4 randomly selected clones after *E. coli* TOP10 transformation with the ligation reaction products. Positive clones, which contain full-length *sf*GFP gene, were indicated (+). DNA fragments were separated into 1.5 % agarose gel where side arrows indicate the expected positions and sizes (in bp) of these fragments as well as the bands of DNA molecular weight marker (M).

3.2. Expression and purification of sfGFP

Production of *sf*GFP as soluble protein was obtained after transformation of *E. coli* BL21 (DE3) Rosetta cells with the confirmed pRSET-*sf*GFP plasmid construct. Cells were grown in LB medium supplemented with antibiotic and protein expression was then induced by IPTG. Purification of *sf*GFP from cytoplasmic extract was done on immobilized-metal affinity chromatography, using Nickel-charged NTA column installed on AKTA prime system. The UV-detector, supplemented with this system, enabled real-time monitoring of the different steps of *sf*GFP purification (Fig. 2A). The protein expression and purification procedures of *sf*GFP were followed by SDS-PAGE separation and coomassie brilliant blue staining (Fig. 2B). A remarkable expression of *sf*GFP could be observed after IPTG induction and incubation for 16 h at 19 °C. Although, expressed protein was totally purified from bacteria cytoplasmic extract by column purification which yielded 90 % pure *sf*GFP. The yield of purified recombinant protein estimably reached 150-200 mg/liter of bacteria culture.

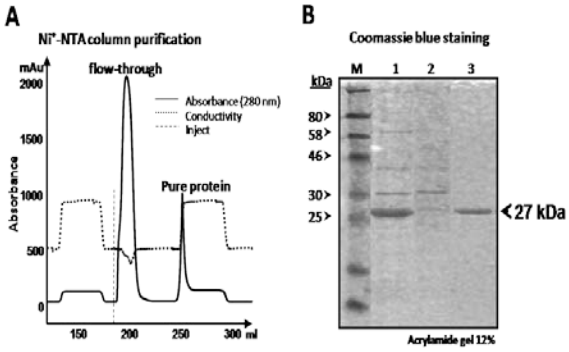

Figure 2. The expression and purification of *sf*GFP
(A) Diagram of purification procedure using Ni^{+}-NTA column installed on FPLC AKTA prime system. Continuous line represents the absorbance of the eluate, different purification steps are shown below and peaks of the flow-through sample and of purified *sf*GFP are indicated. Dashed line represents conductivity of the eluate. (B) SDS-PAGE (acrylamide 12 %) of protein samples from different steps of expression and purification; total cytoplasmic extract after 18 h of IPTG induction (lane 1), flow-through sample from Ni-NTA column (lane 2) and purified *sf*GFP protein (lane 3).

3.3. Rabbit immunization with sfGFP

An adult female rabbit was immunized with four doses of purified *sf*GFP by subcutaneous injections. Blood samples were collected at several time points from the start of the immunization and tested in ELISA against *sf*GFP to evaluate the raise of specific immune response (Fig. 3A). A solid phase ELISA test of the serum dilution (1:5000) prepared from these samples revealed an increase in reactivity toward *sf*GFP after four weeks of immunization (Fig. 3A). At the end of the immunization, titration of rabbit serum (S45) showed a high reactivity since it was still able to detect significantly its antigen at a 10^5-fold dilution (Fig. 3B). From this serum sample (S45), rabbit IgGs were purified by affinity chromatography using protein-A column. An automated purification procedure was established using the AKTA prime system allowing a direct and confirmed purification of the total IgGs from 5 ml serum (Fig. 3C).

3.4. Purification of rabbit anti- sfGFP IgGs

Anti-*sf*GFP was extracted from the total IgGs of the immunized rabbit by affinity chromatography using covalently-immobilized *sf*GFP on a sepharose column. To establish this column, pure recombinant *sf*GFP was coupled by means of the NH2 group to the active NHS group of the resin. After column washing and calibration, total serum IgGs, purified from the previous step, were loaded and unbound IgGs were eliminated by washing and specific anti-*sf*GFP antibodies were recovered from the column by elution with low pH buffer (Fig. 4A). The high capacity of *sf*GFP-column to fix specific IgGs was tested using increasing amounts (2, 5, 10 and 20 mg) of total rabbit IgGs since increasing purified IgG peaks were obtained after each injection. Although, the specificity of this column was confirmed by applying a negative control IgG (20 mg), which did not result in any purification peaks (Fig. 4A).

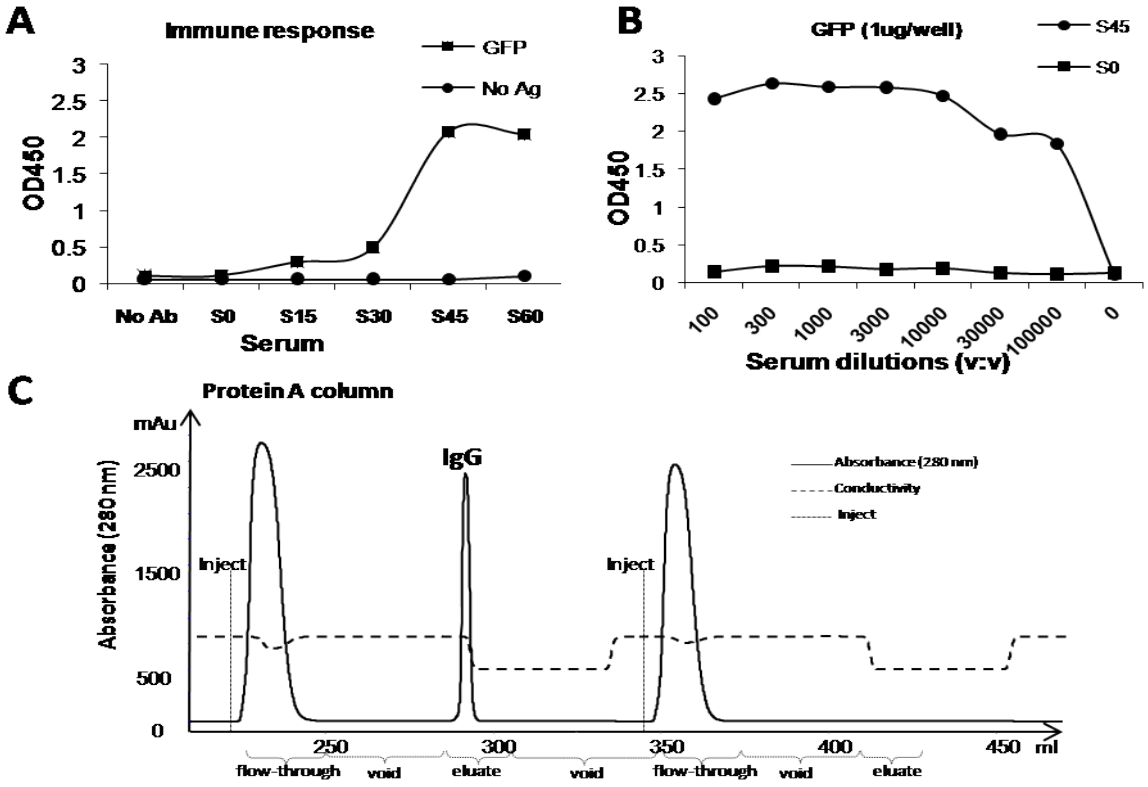

Figure 3. Evaluation of rabbit immune response raised against *sf*GFP (**A**) ELISA test of reactivity against *sf*GFP (0.5 µg/well) of several serum samples taken from the immunized rabbit at several time points from the first injection onwards. Rabbit serums were diluted to 1:5000. Antibody/antigen interaction was detected by goat anti-rabbit HRP conjugated antibody. (**B**) Pure *sf*GFP (0.5 µg/well) was immobilized and detected by ELISA using serial dilutions (v: v) of serum sample before (S0) or after immunization (S45). (**C**) Purification of IgG from rabbit serum by affinity chromatography using AKTA prime FPLC system. Five ml of rabbit serum (S45) was injected (inject 1) onto HiTrap Protein A column and washed with phosphate buffer (void) to remove the unbound proteins (flow-through), before eluting pure IgGs (eluate). A second purification step (Inject 2) was done using the flow-through from the first injection to confirm the total purification of IgGs from the serum.

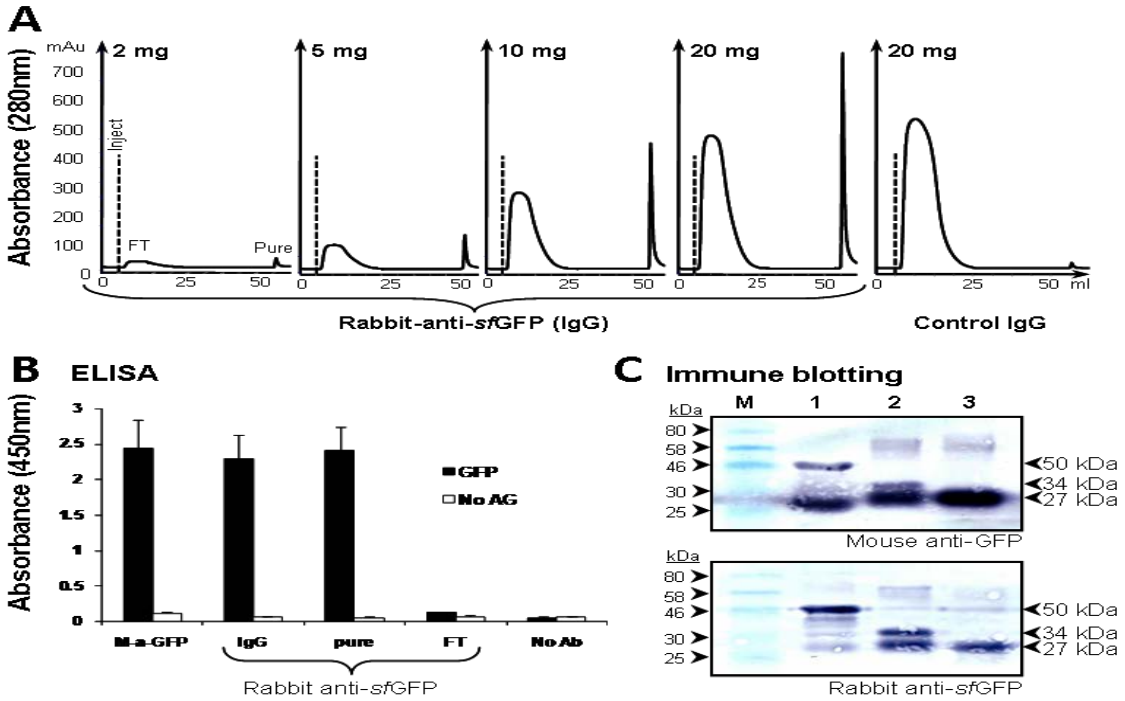

Figure 4. Immunoaffinity chromatography of specific rabbit-anti *sf*GFP IgG (**A**) Affinity *sf*GFP-column (1 ml) was used in chromatography to purify specific antibodies from increasing amounts of IgGs (2 - 20 mg) from the immunized rabbit as well as from control IgG (20 mg). Total antibody sample (5 ml) was loaded in the column (inject) and unbound fraction (FT) was removed by washing and pure anti-*sf*GFP IgGs (P) were recovered from column by elution. (**B**) ELISA test in the absence of antibodies (No Ab) or using a commercial mouse anti-GFP (M-a-GFP) or different IgG samples from the previous purification. Antibodies (1 mg/ml stock) were tested at dilution (1:5000 v:v) in the presence of immobilized *sf*GFP (0.5 µg/well) or in the absence of antigens (No Ag). (**C**) Immunoblotting of three *sf*GFP derivatives (0.2 µg); *sf*GFP-GH (lane 1), *sf*GFP-IGF (lane 2) and *sf*GFP (lane 3) using commercial mouse anti-GFP (Top; 1:1000 v:v) or pure rabbit-anti *sf*GFP IgG (Bottom; 1:1000 v:v).

3.5. Testing of purified anti-sfGFP antibody

In order to evaluate the activity of the purified antibody against its specific antigen, indirect ELISA was performed in *sf*GFP-precoated wells and the bound rabbit IgGs were detected with a goat-anti-rabbit secondary antibody conjugated to HRP (Fig. 4B). As expected, total rabbit and purified IgGs were able to detect efficiently *sf*GFP at high dilution (1:5000 v: v) in a similar way as a commercial mouse anti-GFP antibody. Meanwhile, the flow-through IgGs, which represented the big fraction of the rabbit antibodies during *sf*GFP-affinity purification, failed to bind to *sf*GFP, even at very low dilutions (data not shown). Eliminating this unspecific fraction could be of great importance to reduce the background noise in many immunological applications using this anti-*sf*GFP polyclonal antibody.

Immunoblotting was performed for *sf*GFP and for two other GFP-fused proteins; *sf*GFP-GH and *sf*GFP-IGF (homemade recombinant proteins) using purified anti-*sf*GFP IgG or mouse anti-GFP (Fig. 4C). The fusion proteins contain a protease cleavage site between the fused partners resulting in the presence of a small part of the free 6×His-tagged *sf*GFP even in the purified samples. Comparing to the commercial monoclonal antibody which has a tendency to recognize the free *sf*GFP in the three samples, polyclonal anti-*sf*GFP was able to see to the same extent fused and free *sf*GFP.

4. Discussion

Fusion protein technology represents one of the best solutions to achieve rapid, efficient, and cost-effective protein expression and purification of recombinant proteins (Southward and Surette, 2002). It facilitates purification, enhances protein expression and solubility, chaperones proper folding and reduces protein degradation or toxic effects (Davis *et al*., 1999). Many reports have described the application of GFP fusions in the expression of hard-to-fold proteins (Vasiljevic *et al*., 2006), toxins (Soler-Jover *et al.*, 2004), proteases (van den Berg *et al.*, 2006; Wu *et al*., 2009) and medical short peptides (Skosyrev *et al.*, 2003; Al-Homsi *et al.*, 2012) in both prokaryotic and eukaryotic cell types. This work described the establishment of a high-level protein expression system in *E. coli* using pRSET plasmid, which enables via its T7 promoter the production of soluble s*f*GFP fusions. pRSET-s*f*GFP gives a wide choice for gene insertion through several cloning sites downstream the s*f*GFP gene for a number of widely used and unique-cutting restriction enzymes (BamHI, PstI, KpnI, BglII, NcoI, EcoRI and HindIII), which are followed by a stop codon (TAG). Thus this plasmid provides high yields expression of N-terminal 6×His tagged s*f*GFP alone or in fusion with C-terminal proteins which could be efficiently purified using nickel-charged resin.

This plasmid should enable the production of cytoplasmic GFP which could be fused with low solubility proteins like proteases (Wu *et al.,* 2009), toxic polypeptides (Skosyrev *et al.,* 2003), low stability proteins (Vasiljevic *et al.,* 2006) and membrane proteins (Hsieh *et al.,* 2010).

An advanced upgrade could be done to this plasmid by adding an engineered cleavage site downstream GFP in order to liberate the fused protein after expression and purification. Fusions can be cleaved by either chemical or enzymatic strategies using many well characterized proteases, e.g. Tobacco Etch Virus (TEV) protease (Polayes *et al.,* 1998), Factor Xa protease (Jenny *et al.,* 2003), and Enterokinase (Hung and Chiou, 2000), which can recognize and cut at specific amino acid sequences. Cleavage of the fusion usually is necessary because of the possible interference with the structural or functional properties of the recombinant protein. TEV protease represents the best choice for our system because of its high specificity and activity towards its cutting site (van den Berg *et al.,* 2006). Although, many previous works have described the possibility to produce TEV as soluble recombinant 6×His-tagged protein in *E. coli* (Lucast *et al.,* 2001; Tropea *et al.,* 2009; Wu *et al.,* 2009). In this model, purified s*f*GFP-fused protein could be cleaved with TEV protease and the desired native protein could be recovered after eliminating TEV protease by nickel affinity chromatography together with the 6×His-tagged s*f*GFP portion. Visibility of GFP is the most distinguishable element from other fusion tags (Müller-Taubenberger and Anderson, 2007). Its unique green color (at visible or UV light) allows us to monitor the presence of GFP-fused protein through expression and purification steps even without any measurement apparatus like UV spectrophotometer (Lee, 2009). Another modification could be added to pRSET-s*f*GFP plasmid in order to release the expressed protein outside the cytoplasm, which is important to reduce some cases of protein degradation (Talmadge and Gilbert, 1982). Adding a short sequence coding for a leader peptide, as pelB signal, to the N-terminal of s*f*GFP should enable the expression of the protein in the periplasm of *E. coli*. Protein expression in the oxidized periplasm is important to produce properly folded disulfide-bonded proteins (de Marco, 2009; Dinh and Bernhardt, 2011).

Despite the previously reported low immunogenicity of GFP (Pan *et al.,* 2009), rabbit immunization with expressed and purified s*f*GFP resulted in a high titer immune response (~1/50000). Fusion to GFP is particularly important for the preparation of antibodies against small proteins (<20 kDa) or even medical short peptides, which are not able to induce sufficient immune response by themselves. In fusion structures, s*f*GFP could play the role of a carrier for these small molecules in order to be displayed to the animal immune system and thus the production of their specific antibodies. In the other hand, s*f*GFP fusions which display pathogenic proteins could also be efficiently used in the development of diagnostic kits using fused proteins as immobilized antigens.

Monoclonal anti-GFP antibodies are very specific, however, they are very hard to produce, expensive and target one epitope which can be, under some cases, altered when the s*f*GFP is expressed in fusion with other proteins. However, some of these antibodies can lose their recognition capacity in immunoblotting when protein samples of GFP are denatured (Nakamura *et al.,* 2008). In this work, the main drawbacks of polyclonal antibodies; the high background noise and the low specificity, were surmounted using affinity chromatography purification of polyclonal rabbit IgGs. Recombinant s*f*GFP was covalently immobilized on NHS-activated sepharose column by means of the N-terminus using free amine conjugation chemistry. The affinity chromatography column, which is created by this method, was successfully used to purify specific anti-s*f*GFP antibodies from total rabbit IgGs. FPLC purified polyclonal anti-s*f*GFP IgG represents a valuable tool to detect GFP in many biotechnological techniques. In addition, the development of this pure and specific anti-s*f*GFP rabbit polyclonal antibody if does not replace existing commercial monoclonal antibodies; it could serve as a confirmation method when used, for instance, in immunoblotting or ELISA for the detection of s*f*GFP fusions when no specific antibodies are available for the studied fused protein.

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