Engineering of *Escherichia coli* β-galactosidase for solvent display of a functional scFv antibody fragment

Pilar Alcalá, Neus Ferrer-Miralles[1], Antonio Villaverde*

Institut de Biotecnologia i de Biomedicina and Departament de Genètica i de Microbiologia, Universitat Autònoma de Barcelona, Bellaterra, 08193 Barcelona, Spain

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Abstract Protein engineering allows the generation of hybrid polypeptides with functional domains from different origins and therefore exhibiting new biological properties. We have explored several permissive sites in *Escherichia coli* β-galactosidase to generate functional hybrid enzymes displaying a mouse scFv antibody fragment. When this segment was placed at the amino-terminus of the enzyme, the whole fusion protein was stable, maintained its specific activity and interacted specifically with the target antigen, a main antigenic determinant of foot-and-mouth disease virus. In addition, the antigen-targeted enzyme was enzymatically active when bound to the antigen and therefore useful as a reagent in single-step immunoassays. These results prove the flexibility of *E. coli* β-galactosidase as a carrier for large-sized functional domains with binding properties and prompt the further exploration of the biotechnological applicability of the scFv enzyme targeting principle for diagnosis or other biomedical applications involving antigen tagging.

Key words: Antigen; β-Galactosidase; Protein engineering; Recombinant antibody; Single-chain Fv

1. Introduction

Protein engineering allows a modular design of non-natural polypeptides, in which different functions associated with independent domains from different origins can be combined in a desirable way to serve specific purposes. Examples of such multifunctional hybrid proteins include vehicles for delivery of targeted DNA [1] or functional enzymes [2] and molecular sensors [3]. The *Escherichia coli* β-galactosidase is a homotetrameric enzyme [4] widely employed as a convenient tool in molecular and cell biology. Its tolerance to both terminal fusions [5] and inner peptide insertions [6] has prompted its use as a carrier to stabilize recombinant proteins [7], as a purification tag [8] and as a reporter for analysis of gene expression through gene fusions [9]. The availability of different substrates rendering colored, fluorescent or luminescent components enhances the versatility of this enzyme for fine analytic determinations, simple ELISA tests or in situ determinations of gene expression. In this work, we have explored several tolerant sites of this enzyme, previously identified by insertional mutagenesis and gene fusion [6,10], to display a functional, single-chain antibody fragment (scFv) keeping both the enzymatic activity and the antigen binding properties. The feasibility of this approach would allow the use of this hybrid enzyme for the tagging of specific antigens and among other possibilities, its application as a single molecule reagent in conventional ELISA. The obtained results indicate that the amino-terminus of this enzyme is capable of accepting an antiviral scFv antibody and that the hybrid protein is produced in *E. coli* at a significant yield. The antibody–enzyme chimera specifically recognized the antigen against which the parental antibody was elicited and it was enzymatically active while bound to the antigen.

2. Materials and methods

2.1. Bacterial strains, plasmids, and cloning strategy

The *E. coli* K12 strain MC1061 ((F$^-$, λ$^-$), Δ(*ara*A-*leu*)7697, Δ(*codlac*I)3, *ara*D139, *gal*E15, *gal*K16, *hsd*R2, *mcr*A, *mcr*B, *rel*A1, *rps*L150 (strR), *spo*T1) was used for cloning purposes. Fusion proteins were produced in the protease-deficient *E. coli* strain BL26, a Lac$^-$ derivative of BL21 (*dcm, hsd*S, *gal*, Lon$^-$, OmpT$^-$) [11]. All the recombinant vectors constructed in this work derive from pJLACZ [12], encoding a pseudo-wild type *E. coli* β-galactosidase enzyme whose production is driven by both λ p_L and p_R promoters placed in tandem and controlled by the thermolabile cI857 repressor, encoded by the same vector.

Plasmids pPA278SD6, pPANlacZSD6 and pPAlacZCSD6 were constructed to present the scFv93 protein inserted between residues 279 and 280 (within the activating interface of the enzyme) or fused to either the amino- or carboxy-terminus of β-galactosidase respectively (Fig. 1). scFv93 is a recombinant version of the SD6 antibody, which binds specifically to a main B-cell epitope from foot-and-mouth disease virus (FMDV) from serotype C_1, sited in the VP1 capsid protein [13]. The scFv93-encoding DNA was generated by PCR amplification (driven by the proofreading Deep Vent® DNA polymerase) from plasmid pCASD6 93 [14], using two alternative pairs of complementary oligonucleotides. The pair CSD6S (5′-GGG ATC CCA GGT GAA ACT GCA GC-3′) and CSD6R (5′-GGG ATC CCC GTT TTA TTT CCA GC-3′) was used to obtain a product for ligation into the *Eco*RI-digested vector pJLACZ, resulting in vector pPAlacZCSD6. The pair NSD6S (5′-GGA TCC CAG GTG AAA CTG CAG C-3′) and NSD6R (5′-GGG GAT CCC CGT TTT ATT TCC AGC-3′) was used to obtain a product for ligation into the *Nco*I-digested vector pJLACZ, resulting in vector pPANlacZSD6. For cloning at position 279–280, the product of amplification with the NSD6S-NSD6R pair was digested with *Bam*HI and repaired with Klenow enzyme. Then, the DNA segment was ligated into the *Cla*I-digested vector pJLACZ, also repaired with Klenow enzyme, resulting in the vector pPA278SD6.

2.2. Cultures, protein production and enzymatic assays

Plasmid-containing cell cultures were grown at 28°C and 250 rpm in 200 ml of Luria–Bertani (LB) medium [15] plus 100 μg/ml ampicillin

*Corresponding author. Fax: (34)-93-5812011.
E-mail address: avillaverde@servet.uab.es (A. Villaverde).

[1] Present address: Microbiology Unit, Universitat Pompeu Fabra, Doctor Aiguader 80, 08003 Barcelona, Spain.

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until they reached OD_{550} 0.3, and then were transferred to a water bath warmed at 42°C, where recombinant gene expression was allowed for 3 h at 250 rpm. β-Galactosidase activity of bacterial cultures was assayed as described [16] in samples of 0.1 ml plus 0.9 ml of Z buffer (0.06 M Na_2HPO_4, 0.04 M NaH_2PO_4, 0.01 M KCl, 1 mM $MgSO_4$, pH 7) at 28°C. The reaction was initiated with 200 μl of 4 mg/ml *ortho*-nitrophenyl β-D-galactopyranoside (ONPG) in phosphate buffer [16], and when the yellow color was evident it was stopped with 500 μl of 1 M Na_2CO_3. The absorbance of the samples was read at 414 nm in a Labsystems iEMS Reader MF.

2.3. SDS–PAGE and Western blotting

Producing bacterial cells were harvested and concentrated (40×) by low-speed centrifugation and pellets were resuspended in 3 ml of TM buffer (20 mM Tris–HCl, 10 mM $MgCl_2$, pH 7.2) supplemented with one protease inhibitor cocktail tablet (Roche) per 7 ml of TM buffer. When necessary, soluble and insoluble cell fractions were separated by centrifugation at 16 000×*g* and 4°C for 10 min, and boiled separately in Laemmli buffer [17], loaded on 7.5% SDS–PAGE and blotted onto nitrocellulose sheets. Proteins were immunodetected with anti-β-galactosidase rabbit serum (1/500) obtained against the commercial enzyme. Anti-rabbit antibodies coupled to horseradish peroxidase were obtained from Bio-Rad and used at 1/2000. Bands were developed with H_2O_2 and 4-chloro-1-naphthol as a substrate. Blot images were obtained in a Hewlett-Packard high-resolution scanner and relevant bands were quantified by using Quantity One software from Bio-Rad. Specific activity of soluble PANlacZSD6 was calculated from the enzymatic activity and the fusion protein amount in cell extracts, 2 h after induction of gene expression.

2.4. Peptides and ELISA

The 21-mer linear peptide A21 (YTASARGDLAHLTTTHARHLP) reproduces a segment of the G-H loop from FMDV VP1 protein, the antigenic site A, which includes the antibody SD6 B-cell epitope [13]. The 13-mer control peptide P2 (CSGKLICTTAVPW) reproduces an immune-relevant B-cell epitope from gp41 of HIV [18]. Binding of PANlacZSD6 to peptides was tested in solid phase by sandwich ELISA as follows. Different amounts of peptide A21 or the control peptide P2 from HIV ranging from 0.08 to 100 pmol were incubated overnight at 4°C as coating antigens in microtiter plates. After washing, wells were blocked overnight at 4°C with 300 μl of blocking buffer (5% non-fat dry milk in phosphate-buffered saline containing one protease inhibitor cocktail tablet per 7 ml of buffer). Plates were washed and incubated overnight at 4°C with 100 μl of a 1:1 mixture of blocking buffer and 40× concentrated soluble fraction of BL26/PANlacZSD6 cells, obtained 2 h after induction of gene expression. After washing, rabbit anti-β-galactosidase serum diluted 1/1000 was added and after an overnight incubation at 4°C, plates were washed and further incubated with a solution of goat anti-rabbit IgG (H+L) horseradish peroxidase conjugate (Bio-Rad) under the same conditions. After extensive washing, peroxidase reaction was done by adding a standard solution of H_2O_2, 3-dimethylaminobenzoic acid and 3-methyl-2-benzothiazolinone as substrates (Sigma). The reaction was stopped with 2 M H_2SO_4 and the resulting blue color was read at 620 nm. Monoclonal antibody SD6 at 18.7 ng/μl and protein LACZ in cell extracts prepared as for PANlacZSD6 were used as positive and negative controls respectively for binding to peptide A21. SD6 was detected with a goat anti-mouse IgG horseradish peroxidase conjugate (Bio-Rad).

For the ELISA revealed through the β-galactosidase activity of PANlacZSD6, microtiter plates were incubated overnight at 4°C with peptide A21 or P2 and washing and blocking steps were performed as described above. The incubation with the recombinant protein was done overnight at 4°C. Plates were washed and bound protein was detected by using 4 mg/ml ONPG in Z buffer. The reaction was stopped with 1 M Na_2CO_3 after 2 h incubation at 28°C and the absorbance was read at 414 nm in a Labsystems iEMS Reader MF. All the ELISA tests were done in triplicate.

3. Results

3.1. Bacterial production of ScFv–β-galactosidase fusion proteins

After construction and checking, plasmids pPA278SD6, pPANlacZSD6 and pPAlacZCSD6 were transformed into *E. coli* BL26. Recombinant cells were grown in LB medium without any symptom of toxicity, reaching the same biomass as the control BL26/pJLACZ cultures (not shown). Three hours after the temperature shift, the enzymatic activity was 14 354.2 ± 1191.1, 9477.1 ± 813.7 and 5439.6 ± 124.8 U/ml forpJLACZ-, pPAlacZCSD6- and pPANlacZSD6-bearing cultures respectively, but it was undetectable in BL26/pPA278-SD6 cells. These data suggested differential proteolysis of the fusions, which was confirmed by SDS–PAGE and further Western blot analysis of cell extracts. The full-length form of pPANlacZSD6 was clearly observed, representing between 25 and 30% of the total anti-β-galactosidase-immunoreactive protein, being mainly a 90 kDa degradation fragment (Fig. 2). Traces of a β-galactosidase-like fragment were also visualized. However, no intact forms of PA278SD6 and PAlacZCSD6 were detected at any post-induction time, these proteins occurring only as degradation fragments (not shown). The en-

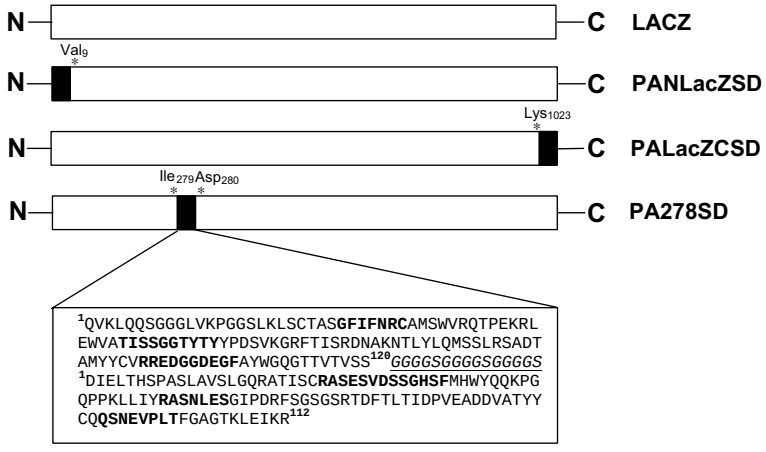

Fig. 1. Schematic representation of the engineered β-galactosidase proteins, indicating the location of the scFv93 insertion in the primary sequence of each construct (black boxes). The inserted segment is 247 amino acids in length. The complementarity determining regions are indicated in bold and the linker in italics and underlined. The numbering of the heavy and light chains is according to the sequence of the parental monoclonal antibody SD6 [13]. The amino acid residues of β-galactosidase, flanking the heterologous stretches, are also indicated by asterisks. The corresponding numbering is according to Fowler and Zabin [31].

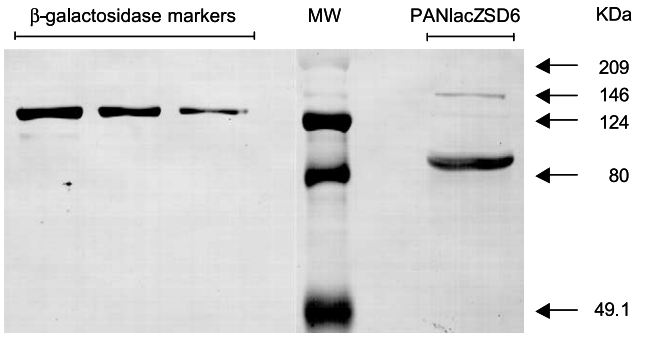

Fig. 2. Western blot analysis of soluble PANlacZSD6 produced in *E. coli* BL26, 2 h after induction of gene expression. The upper band in the right lane corresponds to the full-length PANlacZSD6 (146 kDa). β-Galactosidase markers ranging from 500 ng to 125 ng were loaded in the left lanes. Lane MW indicates molecular weight markers whose apparent masses are indicated by arrows.

zymatic activity associated with PAlacZCSD6 production must then be essentially due to enzymatically active degradation fragments.

Therefore, only the amino-terminal fusion PANlacZSD6 was further characterized regarding both productivity and functionality. After cell fractionation, about 90% of PANlacZSD6 was found in the soluble cell fraction while the rest remained associated with the insoluble cell material. The specific activity was calculated to be 2223.48 ± 92.76 U/μg, similar to that previously reported (2765 ± 347 U/μg) for the parental, pseudo-wild type β-galactosidase LACZ [6]. Two hours after induction of gene expression, the productivity in shaker flasks of the soluble, full-length form of PANlacZSD6 was found to be 1.1 μg/ml culture.

3.2. Specific binding and enzymatic activity of PANlacZSD6

To evaluate the proper solvent exposure and binding specificity of the scFv domain as displayed on the surface of

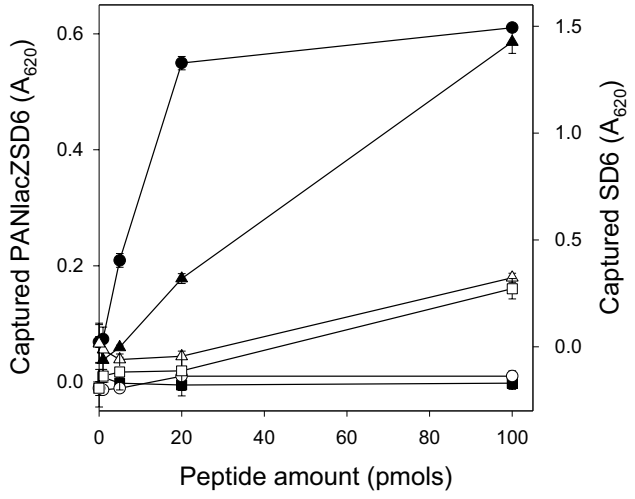

Fig. 3. Binding of protein PANlacZSD6 to peptides A21 (black triangles) and P2 (white triangles) as measured by an anti-β-galactosidase monoclonal antibody. The union of the monoclonal antibody SD6 to A21 (black circles) and P2 (white circles) is also presented. Note that the absolute absorbance values of PANlacZSD6 and SD6 are not comparable as the second antibody is different in both cases. The background binding of LACZ protein to peptide A21 (black squares) and peptide P2 (white squares) is also shown as a control.

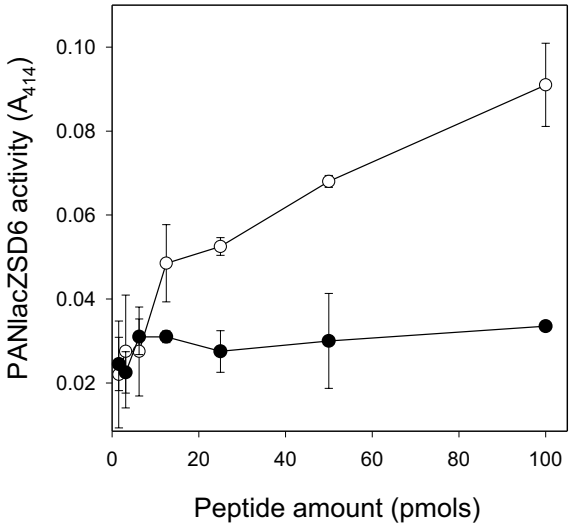

Fig. 4. Binding of protein PANlacZSD6 to peptides A21 (white circles) and P2 (black circles) as measured by the PANlacZSD6 β-galactosidase activity.

PANlacZSD6, a capture ELISA was performed with the A21 SD6 epitope peptide and the SD6-irrelevant P2 peptide as bound antigens. As shown in Fig. 3, PANlacZSD6 but not the parental LACZ protein was retained by A21 (but not by P2), as determined by an anti-β-galactosidase antibody. This fact proved the correct presentation and binding specificity of the recombinant fusion version of SD6. To evaluate if in the bound state PANlacZSD6 was able to retain its enzymatic activity, an additional capture ELISA was performed in which the binding of the fusion was monitored by its enzymatic activity. Fig. 4 shows the increase of β-galactosidase activity retained by growing amounts of A21, demonstrating that the fusion protein is active even when interacting with the antigen through its amino-terminal scFv domain. This result indicates that scFv–β-galactosidase fusions are appropriate tools for molecular targeting and confirms the plasticity of this enzyme for diverse biotechnological purposes.

4. Discussion

Being an enormous tetrameric enzyme, the tolerance of *E. coli* β-galactosidase to both end-terminal protein fusions and inner peptide insertions is remarkable [5,6]. In previous works, we had inserted up to 45-mer peptides in the close vicinity of the active site, maintaining the enzymatic activity of the hybrid proteins [6,10,19]. In most of the identified permissive sites the foreign peptide was solvent-exposed, antigenic and available for ligand–receptor interactions with target molecules [20,21]. However, the proteolytic stability of some of these constructs was seriously impaired by specific foreign stretches [19]. On the other hand, an extensive degradation was also observed in a carboxy-terminal fusion (the equivalent amino-terminal version being much more stable) carrying a 23 kDa heterologous protein [22]. In this work we tried three acceptor sites of the enzyme (namely the two terminal ends and an inner permissive site) for the solvent display of an scFv to construct an antigen-targeted recombinant enzyme (Fig. 1). Among them, only the amino-terminus of β-galactosidase tolerated the engineered protein domain of 247 amino acids reproducing the paratope of the monoclonal antibody SD6.

Although partial degradation of this protein (named PANlacZSD6) was also detected (Fig. 2), around 30% of the β-galactosidase-immunoreactive material occurred in its full-length form. Also, it was mainly produced as a soluble protein and at reasonably high yields, without significant reduction of specific activity when compared with the parental LACZ protein. Therefore, these results support a high tolerance of the β-galactosidase amino-terminus versus the sensitivity of the carboxy-terminus, and prompt the further discarding of positions 279–280 (being permissive to short peptides), to accommodate large protein domains.

On the other hand, protein PANlacZSD6 recognizes specifically the SD6 target antigen (Fig. 3) and maintains its enzymatic activity in the bound state (Fig. 4). This is especially important because the activity of *E. coli* β-galactosidase can be affected by the binding of antibodies directed against either enzyme epitopes [23] or inserted antigenic peptides [19,24]. In addition, the dual activity of PANlacZSD6 (namely antigen binding and catalytic properties) can be extremely useful in the biomedical context for tagging specific antigens. An immediate example is the application of this principle in diagnostic immunoassays through the use of single-chain enzymatic antibodies produced in recombinant organisms. However, the spectrum of possibilities for such an enzyme–antibody fusion protein could also eventually embrace therapeutic purposes.

End-terminal fusions between alkaline phosphatase and antibody fragments have been previously reported for antigen targeting and to circumvent the use of a second, conjugated antibody in ELISA [25–27]. However, *E. coli* β-galactosidase possesses a wider substrate spectrum including ONPG, chlorophenol red-β-D-galactopyranoside, 5-bromo-4-chloro-3-indoxyl-β-D-galactopyranoside, Galacton®, fluorescein di-β-D-galactopyranoside, phenylethyl β-D-thiogalactopyranoside and β-methylumbelliferyl β-D-galactopyranoside, that rendering either colored, fluorescent or luminescent products allows different analytical strategies. In addition, the easiness of its single-step purification chromatography [28] and the permissivity of this enzyme to multiple peptide insertions [29,30] would allow not only a fast, sensitive and flexible enzymatic determination in automated systems but also its further engineering and bioproduction to display additional protein domains for a new combination of desired functions.

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