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Enzymatic functionalization of a nanobody using protein insertion technology

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Abstract

Antibody-based products constitute one of the most attractive biological molecules for diagnostic, medical imagery and therapeutic purposes with very few side effects. Their development has become a major priority of biotech and pharmaceutical industries. Recently, a growing number of modified antibody-based products have emerged including fragments, multi-specific and conjugate antibodies. In this study, using protein engineering, we have functionalized the anti-hen egg-white lysozyme (HEWL) camelid VHH antibody fragment (cAb-Lys3), by insertion into a solvent-exposed loop of the *Bacillus licheniformis* β-lactamase BlaP. We showed that the generated hybrid protein conserved its enzymatic activity while the displayed nanobody retains its ability to inhibit HEWL with a nanomolar affinity range. Then, we successfully implemented the functionalized cAb-Lys3 in enzyme-linked immunosorbent assay, potentiometric biosensor and drug screening assays. The hybrid protein was also expressed on the surface of phage particles and, in this context, was able to interact specifically with HEWL while the β-lactamase activity was used to monitor phage interactions. Finally, using thrombin-cleavage sites surrounding the permissive insertion site in the β-lactamase, we reported an expression system in which the nanobody can be easily separated from its carrier protein. Altogether, our study shows that insertion into the BlaP β-lactamase constitutes a suitable technology to functionalize nanobodies and allows the creation of versatile tools that can be used in innovative biotechnological assays.

Key words: antibody engineering, β-lactamase, multifunctional enzyme, nanobody, protein chimera

Introduction

The ability of immunoglobulins to specifically recognize and bind with high affinity to any type of antigen makes them interesting molecules for medical, diagnostic and scientific purposes. For these reasons, many efforts were devoted to their development and optimization such as the minimization of the antibody size, the improvement of their production or their coupling to diverse molecules. Whole antibodies are complex molecules that consist of heavy and light chains. In addition, N-linked oligosaccharides are also attached to specific domains of the antibody which further increases the degree of complexity of these proteins. N-glycosylation is essential in antibody-dependent cellular toxicity (Kanda *et al.*, 2006; Scallon *et al.*, 2007), complement-dependent cytolysis (Hodoniczky *et al.*, 2005) and for retaining a long serum half-life (Ferrara *et al.*, 2006; Ishino *et al.*, 2013). Thus, controlling glycosylation of antibody by using cell engineering techniques is also a new path of investigation to regulate the Fc function.

More recently, the emergence of protein engineering has led to the generation of tailored antibodies such as modified antibodies or antibody fragments. Modification of antibodies allows the customization of their functionality and physico-chemical properties that consequently broadens their applications. Indeed, in some cases, only the antigen-binding site of the antibody is required and sometimes preferred, this is why a variety of functional antigen-binding antibody fragments such as Fab, Fv, scFv, diabody, triabody, tetrabody and nanobody have been developed (Todorovska *et al.*, 2001; Holliger and Hudson, 2005; Nelson, 2010). This minimization of the antibody size has also facilitated their evolutive affinity maturation, large-scale production and affinity selection using high-throughput techniques such as phage display, ribosome display or yeast display.

In classical antibodies, the antigen-binding site consists of the heavy and light chain variable domains (VH and VL). Each of these domains contains four conserved framework regions and three complementary determining regions (CDRs). The CDRs are hypervariable regions and their variability determines the specificity and the affinity of the antibody. VH and VL domains are both required to form a fully active binding site. In scFv antibody fragments, the VH and VL domains are linked by a hydrophilic and flexible 15-amino acid linker to form a single-chain. The most commonly used linker in scFv is $(Gly_4Ser)_3$ (Huston *et al.*, 1988). To improve the stability and the compactness of scFv fragments, cysteines were introduced at the interface between the VH and VL domains, forming a disulfide bridge that holds the two domains together (Luo *et al.*, 1995). This approach gave rise to mono- and bispecific diabodies (Holliger *et al.*, 1993).

Despite the numerous advances in the field, the large-scale expression of scFv sequences with low-cost expression systems (e.g. *Escherichia coli*) often results in the production of aggregated proteins. In 1993, Hamers-Casterman *et al.* discovered that *Camelidae* produce functional immunoglobulins as homodimers of only heavy chains, devoid of light chains (Hamers-Casterman *et al.*, 1993). The presence of these single-domain antibodies was also identified in cartilaginous fishes such as sharks (Flajnik and Dooley, 2009). It was also observed that these antibodies have an authentic antigen-binding repertoire and can be induced in llamas or dromedaries with a variety of protein antigens. Sheriff and Constantine showed that the N-terminal variable region (referred to as VHH domain or nanobody), which is connected to the two constant domains (CH2 and CH3) in the full-length heavy chain, constitutes the minimum-sized antigen-binding domain (≈12–15 kDa) (Sheriff and Constantine, 1996). Compared with conventional antibodies and scFv fragments, it corresponds, respectively, to a 10- and 2-fold reduction of the molecular size without altering the antigen-binding affinity as well as no required domain paring. The ability of VHH to recognize cryptic epitopes and enzyme active sites has been attributed to their smaller size and an extended CDR3 loop which both contribute to reduce steric hindrance and to make insertion into such sites easier (Stijlemans *et al.*, 2004; Thanongsaksrikul *et al.*, 2010).

Antibody conjugation is another important aspect of antibody modification and has significantly broadened their applications. Various functional molecules including fluorescent dyes, biotin and enzymes such as the horseradish peroxidase, the alkaline phosphatase, the β-galactosidase or the β-lactamase can be conjugated to antibodies. The generated functionalized antibodies were shown to be extremely useful tools to probe many biologically and chemically important molecules *in vitro* or *in vivo* (Haugland, 2001). Among many other applications, these antibody conjugates have become highly potent molecules in cellular imaging (East *et al.*, 2014) and cancer therapy (Chari *et al.*, 2014).

In the context of antibody conjugation, β-lactamases have emerged as very suitable enzymes. Initially used as biochemical markers for identification of β-lactam antibiotics, β-lactamases have become widely used over the last decade. Indeed, class A β-lactamases in particular are attractive molecules because they are monomeric, very stable and of small size (Philippon *et al.*, 1998). Their three-dimensional structures are well-defined and they are considered as very efficient enzymes (Matagne *et al.*, 1999). In addition, among all the enzymes used for therapeutic purposes, β-lactamases are particularly interesting for several reasons: (i) they do not interfere with the biological function of most of the proteins to which they might be fused, (ii) they do not have any endogenous counterparts in mammalian cells (Naqvi and Singh, 2007) and (iii) finally, non-immunogenic β-lactamases have been engineered (Harding *et al.*, 2005). Furthermore, a large variety of β-lactam substrates are currently available including chromogenic β-lactams for immunoassays, fluorescent membrane-permeant β-lactams that allow cell imagery, flow cytometry and gene expression assay, and non-toxic β-lactam prodrugs for antibody-directed enzyme prodrug therapy (ADEPT) (Bebrone *et al.*, 2001; Vrudhula *et al.*, 2003; Lee *et al.*, 2005, Naqvi and Singh, 2007) that is currently one of the most promising strategy for cancer treatment. Briefly, in ADEPT, monoclonal antibodies are covalently linked to a β-lactamase and are targeted to tumor cells where they deliver toxic agents (Deonarain and Epenetos, 1994). The antibody part of the conjugate allows the specific delivery to tumor cells via the recognition of tumor associated antigens where the β-lactamase part catalyzes the activation of a prodrug at the site of tumor with high specificity (Deonarain and Epenetos, 1994). Non-toxic β-lactam prodrugs (O'Callaghan *et al.*, 1972; Gao *et al.*, 2003; Porter *et al.*, 2008) have been developed and most of them consist in cephalosporins with 3′ leaving groups that are released upon hydrolysis of the β-lactam ring. Various toxic compounds can thus be released from cephalosporin prodrugs, including nitrogen mustard and vinca alkaloid derivatives. This technology has been successfully validated *in vitro* with different antibodies-β-lactamase conjugates as well as different tested prodrugs (Muraro *et al.*, 1988; Meyer *et al.*, 1992; Svensson *et al.*, 1992; Roberge *et al.*, 2006).

In this study, we describe the functionalization of the previously characterized hen-egg-white lysosyme (HEWL) cAb-Lys3 (Saerens *et al.*, 2005) nanobody by insertion into the *Bacillus licheniformis* β-lactamase BlaP as a proof of concept to conjugate antibody fragments.

In the previous studies (Chevigne *et al.*, 2007; Vandevenne *et al.*, 2007; Ruth *et al.*, 2008; Vandevenne *et al.*, 2008), we showed that various protein fragments (up to 8 kDa) can be inserted into a solvent-exposed loop of the *B.licheniformis* class A β-lactamase called BlaP (30 kDa) and the resulting chimeric proteins conserve both the β-lactamase activity and the biological function of the inserted protein fragment. The loop chosen for protein fragment insertion is located far away from the active site and connects helix 8 to helix 9 (between residues Asp 197 and Lys 198) at the interface between the α domain and the α/β domain of the β-lactamase. It has to be noticed that we have chosen this particular class A β-lactamase because this enzyme is a natural protease-insensitive protein adapted to the presence of various and numerous proteases that are co-expressed by *B.licheniformis* (Filee *et al.*, 2002; Brans *et al.*, 2004) and is therefore much more resistant to proteolysis than its counterparts in other prokaryotic organisms such as TEM-1 produced by *E.coli*.

Herein, we demonstrate that our protein insertion strategy allows the functionalization of cAb-Lys3 without altering its binding activity. We also showed that the presence of the enzymatic β-lactamase activity

presents valuable advantages when our technology is implemented in phage display, potentiometric biosensor and drug screening assays.

Materials and methods

Expression and purification of the hybrid β-lactamases: BlaP cAb-Lys3 and BlaP Thb/cAb-Lys3/Thb

To achieve production of the hybrid β-lactamases harboring a polyhistidine tag, *E.coli* JM109 cells transformed with the plasmid encoding the BlaP cAb-Lys3 or BlaP Thb/cAb-Lys3/Thb fusion proteins were grown in Terrific Broth supplemented with 75 mg/ml spectinomycin and 10 mg/ml ampicillin at 37°C. Cells from an overnight culture (1 l) were harvested by centrifugation (9000 *g* for 15 min) and re-suspended in 40 ml of TES (20% sucrose, 30 mM Tris–HCl, 5 mM EDTA, pH 8) at 37°C. The bacterial suspension was placed under stirring at 37°C for 10 min. Cells were harvested by centrifugation (9000 *g* for 15 min) and the pellet re-suspended in 100 ml of 5 mM Mg_2SO_4 at 4°C. The bacterial suspension was stirred at 4°C for 10 min. The supernatant containing the periplasmic proteins was harvested by centrifugation (13 000 *g* for 20 min) and diluted with three volumes of 50 mM phosphate buffer, pH 7.4 (buffer A). The periplasmic proteins were loaded on a HisTrapTM Chelating HP column (GE healthcare) equilibrated in buffer A. The column was successively washed with buffer A + 2 M NaCl and buffer A + 10 mM imidazole. The hybrid proteins were eluted by an imidazole linear gradient (10–500 mM imidazole) in buffer A. Fractions containing the purified hybrid proteins were pooled and dialyzed against buffer A.

Thrombin cleavage of BlaP Thb/cAb-Lys3/Thb

Thrombin (Novagen) cleavage was performed in the provided buffer (20 mM Tris–HCl pH 8.4, 150 mM NaCl, 2.5 mM $CaCl_2$) at 25°C overnight. Hundred units of protease were used to digest 1 mg of purified BlaP Thb/cAb-Lys3/Thb in a final volume of 1 ml. To eliminate the N- and C-terminal fragments of the β-lactamase BlaP that remain self-assembled by non-covalent interactions, the sample was loaded on HisTrapTM Chelating HP column. Isolated cAb-Lys3 was collected in the flow through.

β-Lactamase enzymatic assays

The kinetic parameters of the purified bifunctional hybrid β-lactamases were determined using nitrocefin in 50 mM phosphate buffer at pH 7. The initial rate of hydrolysis was monitored at 482 nm. The K_M and k_{cat} values were calculated by fitting the data to the Henri–Michaelis equation and its linearized form according to the Hanes transformation (Frere, 1973).

Lysozyme enzymatic assays

Lysozyme activity inhibition was determined using the DQ™ lysozyme assay kit (Molecular Probes) as substrate. This fluorescence-based assay measures lysozyme activity on *Micrococcus lysodeikticus* cell walls, which are labeled with fluorescein to such a degree that the fluorescence is quenched. Lysozyme action relieves this quenching, yielding a significant increase in fluorescence that is proportional to lysozyme activity. Fluorescence measurements were recorded according to the manufacturer's instructions using a SML-AMINCO Model 8100 spectrofluorimeter (Spectronic Instruments) in a 1 cm optical path length quartz cuvette. The excitation and emission wavelength were 494 and 518 nm, respectively.

Blap cAb-Lys3 interactions studies using surface plasmon resonance

The binding of BlaP cAb-Lys3 to HEWL was analyzed by surface plasmon resonance (SPR) on a Biacore 3000 (GE healthcare) and amine coupling was used to immobilize HEWL on a CM5 sensor chip. The coupling of HEWL was performed as described by the manufacturer using the standard N-ethyl-N′-(3-dimethylaminopropyl)-carbodiimide (EDC) and N-hydroxy-succinimide (NHS) procedure. The CM5 sensor was activated by injecting 70 µl of a solution of 1:1 400 µM NHS and 100 µM EDC at 10 µl/min. This was followed by an injection of HEWL to obtain ~260 RU of coverage at 10 µl/min. The remaining activated functional groups on the sensor chip were blocked by an injection of 70 µl of 1 M ethanolamine at 10 µl/min. Regeneration steps were performed with a 1 min-pulse of 50 mM NaOH 1 M NaCl. All experiments were conducted at 25°C with a flow rate of 10 µl/min using phosphate-buffered saline (PBS) (20 mM sodium phosphate, 50 mM NaCl, pH 7.4) as the running buffer, under these conditions the observed mass transfer effects were negligible. The sensorgrams were analyzed and fitted to a homogeneous 1:1 association model using the BIAevaluation 4.1 software (Biacore, GE Healthcare, USA).

Enzyme-linked immunosorbent assay

The enzyme-linked immunosorbent assay (ELISA) was performed on Maxisorp® microplate strips (Nalge Nunc International). Seventy-two nanograms of HEWL (5 pmol) or an unrelated protein (used as a negative control) diluted in carbonate buffer (50 mM $NaHCO_3$, pH 9.6) was coated per well for 16 h at 4°C. The wells were washed three times with phosphate-buffered saline with tween 20 (PBST) (150 mM NaCl, 50 mM sodium phosphate, pH 7.5 and 0.05% Tween 20) and blocked for 16 h at 4°C with 10% casein hydrolysate in PBS (150 mM NaCl, 50 mM sodium phosphate, pH 7.5). After three successive washings with PBST, 5 pmol/well of BlaP cAb-Lys3 was added to the wells and incubated for 30 min at 37°C. Unbound protein was removed by six successive washing steps with PBST (150 mM NaCl, 50 mM sodium phosphate, pH 7.5 and 0.1% Tween 20). The revelation step was performed by measuring the immobilized β-lactamase activity following the hydrolysis of 100 µM nitrocefin in PBS at 482 nm after 15 min of incubation. The experiment was realized in triplicates.

Competitive ELISA

The competitive ELISA was performed on Maxisorp® microplate strips (Nalge Nunc International). Seventy-two nanograms (5 pmol) of lysozyme were incubated with different amounts of N,N′,N″-triacetylchitotriose ($GlcNac^3$): 25 pmol (5×), 50 pmol (10×), 500 pmol (100×), 5 nmol (1000×), 50 nmol (10 000×), 250 nmol (50 000×) and 500 nmol (100 000×) (100 µl/well) for 30 min at 37°C in 100 µl of carbonate buffer (50 mM $NaHCO_3$, pH 9.6). The mixtures were coated on a microwell plate for 16 h at 4°C. The microplate was then washed three times with PBST (150 mM NaCl, 50 mM sodium phosphate, pH 7.5 and 0.05% Tween 20) and blocked for 16 h at 4°C with 10% casein hydrolysate in PBS (150 mM NaCl, 50 mM sodium phosphate, pH 7.5). After three successive washings with PBST, 5 pmol/well of BlaP cAb-Lys3 was added to the wells and incubated for 30 min at 37°C. Unbound protein was removed by six successive washing steps with PBST (150 mM NaCl, 50 mM sodium phosphate, pH 7.5 and 0.1% Tween 20). The revelation step was performed by measuring the immobilized β-lactamase activity following the hydrolysis of 100 µM nitrocefin in PBS at 482 nm after 15 min of

incubation. For this experiment, a negative control was realized using lactose (500 nmol) as the competitor molecule, which does not bind to the lysozyme active site.

Construction of the fdtet BlaP cAb-Lys3 phagemid

The *cAb-Lys3* gene fragment was ligated in the SmaI-digested fdtet BlaP/SmaI phagemid described previously (Chevigne *et al.*, 2007). The ligation product was transformed into *E.coli* TG1 cells (Stratagene). Electrotransformed cells were recovered in super optimal catabolite repression medium for 1 h at 37°C and plated on luria-bertani (LB) agar medium supplemented with 7.5 µg/ml tetracycline and 10 µg/ml ampicillin.

fdtet BlaP cAb-Lys3 phage expression and preparation

The transformants were picked up and suspended in LB liquid medium supplemented with 7.5 µg/ml tetracycline and 10 µg/ml ampicillin. Phages were amplified at 28°C for 16 h and recovered from the culture supernatant by two polyethylene glycol precipitations. After the first precipitation, a filtration through a 0.45 µm filter was performed to eliminate membrane fragments. Phages were suspended in tris-buffered saline (TBS) (50 mM Tris, 150 mM NaCl at pH 7.4) and the phage concentration was estimated by measuring the absorbance at 265 nm.

Phage ELISA

The ELISA was performed on Maxisorp® microplate strips (Nalge Nunc International). One microgram of HEWL diluted in carbonate buffer (50 mM $NaHCO_3$, pH 9.6) was coated per well for 16 h at 4°C. After washing with tris-buffered saline with tween 20 (TTBS) (TBS with 0.5% Tween 20), the wells were blocked with 10 mg/ml BSA in TTBS for 1 h at 37°C. Then, 10^{12} phage particles in 100 µl were added per well and incubated overnight at 4°C. Unbound phages were removed by three successive washing steps with TTBS. The revelation step was performed by measuring the immobilized β-lactamase activity following the hydrolysis of 100 µM nitrocefin in 50 mM phosphate buffer pH 7.4 at 482 nm after 30 min of incubation.

Biosensor experiments

Electrode preparation

Glassy carbon substrates (Carbone Lorraine) were washed in ultrasonic bath with heptane P.A. and methanol P.A. then dried under vacuum before use.

Polymerization of aniline on the electrode surface

Most polyaniline syntheses were carried out by chronopotentiometry at 0.03 mA/cm^2 during 1250 s (E varying between 0.71 and 0.73 V) using a 0.1 M aniline solution in 1 M $HClO_4$ with an autolab PGSTAT30 potentiostat/galvanostat in a classical three compartment-cell carefully kept under nitrogen. In order to obtain the neutral emeraldine films, the polyaniline-coated carbon was immersed in an ammonia solution to remove all the doping counter-ions.

Binding assays performed on the biosensor platform

HEWL was coated onto the PANI (polyalanine) surface at a concentration of 4 µg/ml in PBS (50 mM sodium phosphate, pH 7.3, 150 mM NaCl) overnight at 4°C. This incubation was followed by three washing steps with PBS. Next, the electrodes were incubated for 1 h at room temperature in a blocking buffer before another washing step with PBS. BlaP cAb-Lys3 solutions were diluted at the appropriate concentration in PBS buffer containing 1% of casein hydrolysate and applied onto the electrode. After antigen–antibody reaction, the biosensor was washed extensively with PBS before being transferred to the flow cell containing 0.1 M NaCl. When a steady state was reached, the sensor response was initiated by addition of a benzylpenicillin solution to reach a final concentration of 3 mM. The 2 M KCl calomel electrode was used as a reference. The potentiometric values were monitored by a digital multimeter (METRAHit 22M, GMC instruments).

Results

Synthesis of the BlaP cAb-Lys3 and BlaP Thb/cAb-Lys3/Thb hybrid proteins

The synthetic gene-fragment encoding cAb-Lys3 was inserted into the permissive insertion site of the *blaP* gene described in the previous studies (Vandevenne *et al.*, 2007; Vandevenne *et al.*, 2008). Two genetic constructs were generated. These two plasmids are *E.coli* expression vectors that allow constitutive periplasmic expression of the hybrid proteins BlaP cAb-Lys3 and BlaP Thb/cAb-Lys3/Thb (Fig. 1). BlaP Thb/cAb-Lys3/Thb differs from BlaP cAb-Lys3 by the presence of two thrombin-cleavage sites that surround the BlaP-insertion site allowing the cleavage of cAb-Lys3 from BlaP. *E.coli* JM109 cells transformed with both plasmids were able to grow in the presence of 10 µg/ml ampicillin indicating the expression of correctly folded, soluble and functional hydrid β-lactamase, in the periplasmic space.

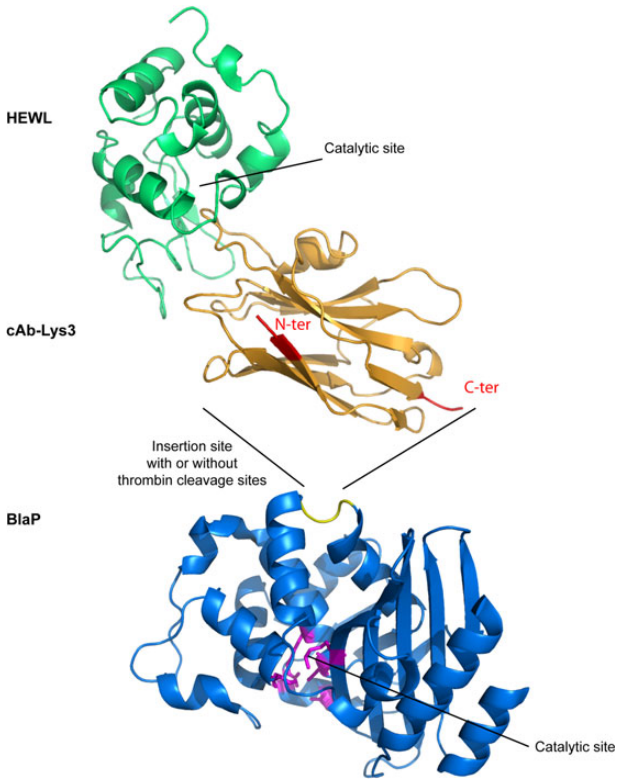

Fig. 1 Representation of BlaP cAb-Lys3 interacting with HEWL. BlaP is shown in blue, cAb-Lys3 in orange and HEWL in green. This figure was obtained by combining the tridimensional structures of BlaP (PDB ID: 4BLM) and the cAb-Lys3/HEWL complex (PDB ID: 1MEL). The N- and C-terminal parts of cAb-Lys3 are indicated in red. The β-lactamase loop used for the insertion is highlighted in yellow. A two-residue linker was used on each side of the insertion site as follow: N-t BlaP/Thr-Gly/cAb-Lys3/His-Cys/C-t BlaP.

After overnight expression in *E.coli*, the two hybrid proteins were extracted from the periplasm and purified to homogeneity on the Profinia IMAC purification system using the BlaP C-terminal polyhistidine tag. After purification, 8 mg of BlaP cAb-Lys3 and 12 mg of BlaP Thb/cAb-Lys3/Thb were obtained per liter of culture.

In the first part of our study, we have focused our study on the BlaP cAb-Lys3 construct to validate the use of the β-lactamase as a carrier protein for nanobody insertion. Later on, we have used the hybrid protein BlaP Thb/cAb-Lys3/Thb to show that our technology also allows the isolation of the inserted nanobody in a simple manner.

Enzymatic characterization of the hybrid β-lactamase BlaP cAb-Lys3

The kinetic parameters of nitrocefin hydrolysis (a chromogenic β-lactam substrate) of BlaP cAb-Lys3 were measured and compared with those observed for the engineered BlaP without any inserted protein. As shown in Table I, the catalytic parameters (K_M and k_{cat}) measured for the hybrid protein and the native β-lactamase are in the same value range, therefore we can conclude that the insertion of cAb-Lys3 into BlaP has a minor impact on its enzymatic activity and that the hybrid β-lactamase retained a good efficiency to hydrolyze β-lactams. This also suggests that the polypeptide insertion does not induce any significant structural perturbations at the level of the β-lactamase active site and therefore we can reasonably assume that the carrier protein remains correctly folded.

Table I. Comparison of the kinetic parameters of the hybrid protein BlaP cAb-Lys3 and the native β-lactamase

	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($\mu M^{-1}\ s^{-1}$)
BlaP	40 ± 2	490 ± 2	11.7 ± 0.1
BlaP cAb-Lys3	46 ± 12	435 ± 31	9.5 ± 3.1

These parameters were determined by measuring initial rates of nitrocefin hydrolysis at 482 nm. The native β-lactamase corresponds to the engineered β-lactamase that includes the insertion site but without any inserted protein fragment.

Affinity and inhibition activity of BlaP cAb-Lys3 for HEWL

The ability of soluble BlaP cAb-Lys3 to bind specifically to HEWL was first suggested from ELISA experiments in which specific interaction between the hybrid protein and HEWL was demonstrated by comparing the immobilized β-lactamase activity on a HEWL-coated microwell plate in the presence of β-lactamase with or without inserted nanobody. No cross-reactivity was measured with unrelated antigens (Fig. 2). These results suggested that coupling a nanobody to a β-lactamase generates an efficient tool that could be implemented in immunoassays or in drug screening assays.

To further confirm the binding of the hybrid protein to HEWL, we also used SPR spectroscopy. The kinetic association (k_a) and dissociation (k_d) constants were measured and the experimental data were fitted using a simple 1:1 binding model. The values presented in Table II indicate that k_a and k_d of the interaction are $3 \times 10^3\ M^{-1}\ s^{-1}$ and $2.2 \times 10^{-4}\ s^{-1}$, respectively. According to these values, the deduced equilibrium dissociation constant (K_D) of BlaP cAb-Lys3 for HEWL is 72 nM which is 6.5-fold higher than the one calculated for the isolated cAb-Lys3 by Dumoulin *et al.* (2002). This suggests that the insertion of cAb-Lys3 into BlaP slightly reduces its association rate with immobilized HEWL. Nevertheless, this effect is partially compensated by a 10-fold decrease of its dissociation rate. However, this small discrepancy observed in the measured binding parameters could also be explained by the fact that the buffers used for these two experiments were different (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.005% surfactant P20, pH 7.4 for the isolated cAb-Lys3 (Dumoulin *et al.*, 2002) and 20 mM sodium phosphate, 50 mM NaCl, pH 7.4 for BlaP cAb-Lys3 in this study). In addition, the molecular sizes of the two analytes are also relatively dissimilar (15 kDa for cAb-Lys3 and 45 kDa for BlaP cAb-Lys3), which could also account for the difference observed in the calculated K_D.

To further confirm that after insertion into the carrier protein, cAb-Lys3 remains folded and retains its ability to bind to its target protein and also inhibits its activity, we measured the inhibition parameters of BlaP cAb-Lys3 on the enzymatic activity of HEWL. The Dixon plot (1/*V* vs. inhibitor concentrations) presented in Fig. 3 indicates that BlaP cAb-Lys3 exhibits a simple competitive inhibition of

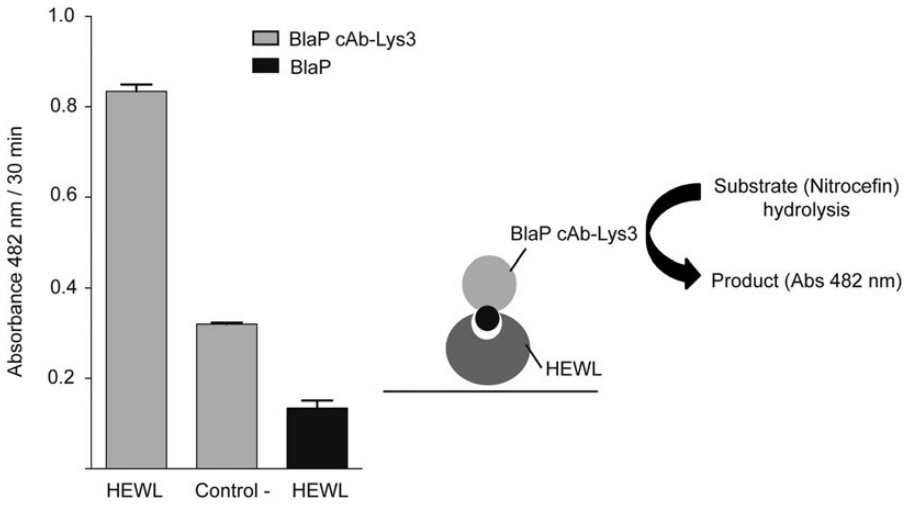

Fig. 2 ELISA showing the specific interaction of BlaP cAb-Lys3 to HEWL. Purified BlaP cAb-Lys3 (gray bars) was added to wells coated with HEWL or a negative control performed with an irrelevant protein coated on the wells. Unbound protein was removed by successive washings with PBST. The bars represent the measured absorbance of the nitrocefin hydrolysis product at 482 nm. These values are directly proportional to the immobilized β-lactamase activity. The experiment was performed in triplicate. An additional control experiment was performed with the purified parental β-lactamase BlaP (black bar) on wells coated with HEWL.

Table II. Kinetic rate constants for association (k_a) and dissociation (k_d), and equilibrium dissociation constant (K_D) of BlaP cAb-Lys3 for HEWL, determined using SPR

Ligand	Analyte	k_a (M^{-1} s^{-1})	k_d (s^{-1})	K_D (nM)
HEWL	cAb-Lys3	1.8×10^{5}*	2.0×10^{-3}*	11*
HEWL	BlaP cAb-Lys3	3.0×10^{3}	2.2×10^{-4}	72

Values indicated by asterisks (*) were reported by Dumoulin *et al.* (2002). A control experiment was realized using BlaP without insert as the analyte and did not show any detectable interaction (data not shown).

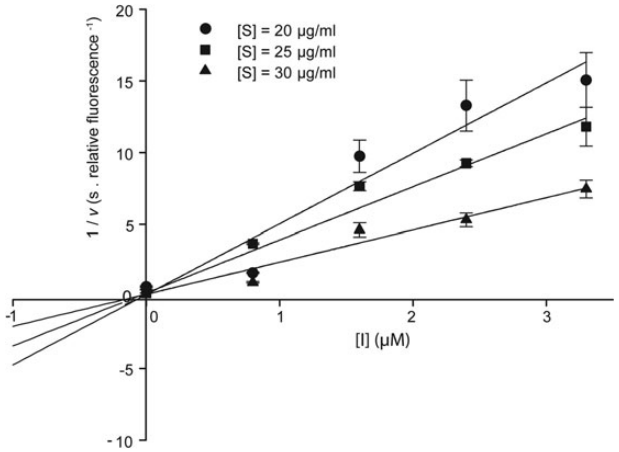

Fig. 3 Dixon plots showing the inhibition of HEWL activity by BlaP cAb-Lys3. Three different substrate concentrations ((circle) 20 µg/ml, (square) 25 µg/ml, (triangle) 30 µg/ml of DQ™ lysozyme substrate, fluorescein conjugate, Molecular Probes) were mixed with samples containing 0.8, 1.6, 2.4 and 3.3 µM of BlaP cAb-Lys3. Lysozyme concentration was kept constant during this experiment and pre-incubated with the different inhibitor dilutions. Initial hydrolysis rates were calculated by measuring the increase in fluorescence (the excitation and emission wavelengths were 494 and 518 nm, respectively). The extrapolated K_i is 40 ± 20 nM.

HEWL and that the extrapolated K_i value (40 ± 20 nM) is in the same value range compared with the one calculated for the isolated cAb-Lys3 (50 nM) (Transue *et al.*, 1998). This result further confirms that the presence of BlaP has very low impact on the ability of cAb-Lys3 to bind HEWL.

Purification of the isolated cAb-Lys3 from BlaP Thb/cAb-lys3/Thb

In previous studies, we showed that the N-terminal and C-terminal parts of BlaP stay in close interaction after cleavage of BlaP Thb/Insertion site/Thb with thrombin and that this property can be used to isolate any protein fragment inserted in between the two cleavage sites (Vandevenne *et al.*, 2008). In the present study, we have adopted a similar strategy to isolate cAb-Lys3 from its carrier protein using a simple two-step procedure. First, we have generated a BlaP Thb/cAb-Lys3/Thb construct and cleaved the purified protein with thrombin. Then, the generated BlaP fragments were depleted by re-loading the sample on a HisTrap Chelating HP column and the isolated cAb-Lys3 was recovered in the flowthrough as illustrated in Fig. 4A and B. This simple strategy has allowed us to retrieve isolated cAb-Lys3 that can be used and characterized as an isolated domain.

It has to be noticed that the band observed around 35 kDa inn Fig. 4B corresponds to the thrombin used for cleavage. Indeed, for this particular construct (BlaP Thb/cAb-Lys3/Thb), a significant amount of protease was required to obtain full cleavage. However, we have not optimized the cleavage conditions or modified the amino acid composition nearby the cleavage sites that could have made the cleavage much more efficient. In addition, commercially available His-tagged thrombin can be used for this cleavage and can be easily removed from the sample when loaded on the HisTrap Chelating column.

Display of BlaP cAb-lys3 at the surface of phage

In previous studies, we reported that libraries of hybrid β-lactamases can be displayed on the surface of phage particles in fusion with the minor coat protein pIII without altering the phage infectivity and that the immobilized β-lactamase activity on a target ligand was useful to monitor phage enrichment during the successive selection rounds (Vandevenne *et al.*, 2007). The phage ELISA presented in Fig. 5 shows the specific interaction measured with fdtet phage displaying BlaP cAb-Lys3 on HEWL. This result shows that phage particles can successfully display cAb-Lys3 inserted into BlaP and therefore that this technology can be implemented for nanobody library screening and affinity maturation.

Potentiometric biosensor assay

One of the characteristics of β-lactam is that their hydrolysis induces the release of protons, which was demonstrated to be important enough to create a change of electrode potential (ΔU) between two electrodes and that the time response of the sensor was very fast upon penicillin hydrolysis and protons production. The amplitude and the kinetics of the ΔU were dependent on the β-lactamase substrate, the identity and the concentration of β-lactamase (Sergeyeva *et al.*, 1999; Gaudin, 2001) immobilized on the electrode. In this study, we have used a similar experimental setup (presented in Fig. 6A) to further demonstrate that the hybrid β-lactamase technology, when combined to nanobodies, can be implemented in potentiometric biosensors. Benzylpenicillin was used as a substrate because of its high solubility and its efficient hydrolysis by BlaP. The biosensor assays presented in Fig. 6B indicate that the specific interaction of BlaP displaying cAb-lys3 with HEWL can be monitored with the biosensor by measuring the immobilized β-lactamase activity. In contrast, no signal could be observed when the experiment was realized with BlaP without any inserted peptide.

Competition binding assay

We have also performed a competitive ELISA presented in Fig. 7, to show that $GlcNAc^3$, which was shown to inhibit HEWL (Vollan *et al.*, 1999), can compete with BlaP cAb-Lys3 to bind to the catalytic site of HEWL. In practice, we have incubated HEWL with different amounts of $GlcNAc^3$ and then coated the mixtures on a microwell plate. Next, BlaP cAb-Lys3 was added to the wells and after adequate washing steps, the immobilized β-lactamase activity was measured. As shown in Fig. 7A, $GlcNAc^3$ could efficiently inhibit the interaction between the hybrid protein and HEWL. We also fitted these data using the classical exponential equation (one phase decay, see Fig. 7B), the derived half-time of the fit corresponds to the IC_{50}. The calculated mean and standard deviation associated with three independent experiments is 961 ± 711 nM. It has to be noticed that according to

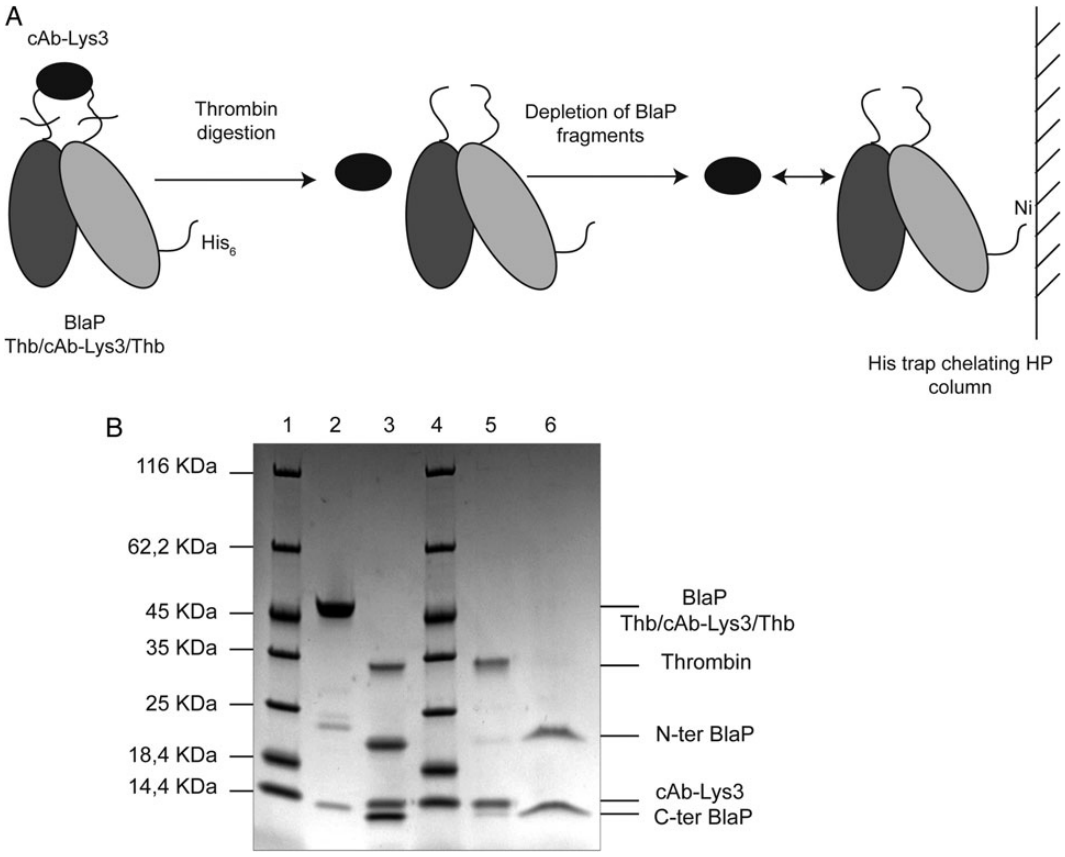

Fig. 4 Representation of the two-step procedure used to purify the isolated cAb-Lys3. (**A**) Scheme representing the experimental setup that allows the isolation of cAb-Lys3. (**B**) SDS–PAGE analysis showing a thrombin-cleavage assay performed on purified BlaP Thb/cAb-Lys3/Thb. Lanes 1 and 4 correspond to the protein molecular weight marker (Fermentas). Lane 2 corresponds to uncleaved BlaP Thb/cAb-Lys3/Thb. Lane 3 corresponds to BlaP Thb/cAb-Lys3/Thb submitted to thrombin cleavage. Lane 5 represents the isolated cAb-Lys3 and thrombin collected in the flow through after loading the sample on HisTrap™ Chelating HP. Lane 6 corresponds to the N- and C-terminal fragments of BlaP Thb/cAb-Lys3/Thb immobilized on the HisTrap™ column and eluted with imidazole.

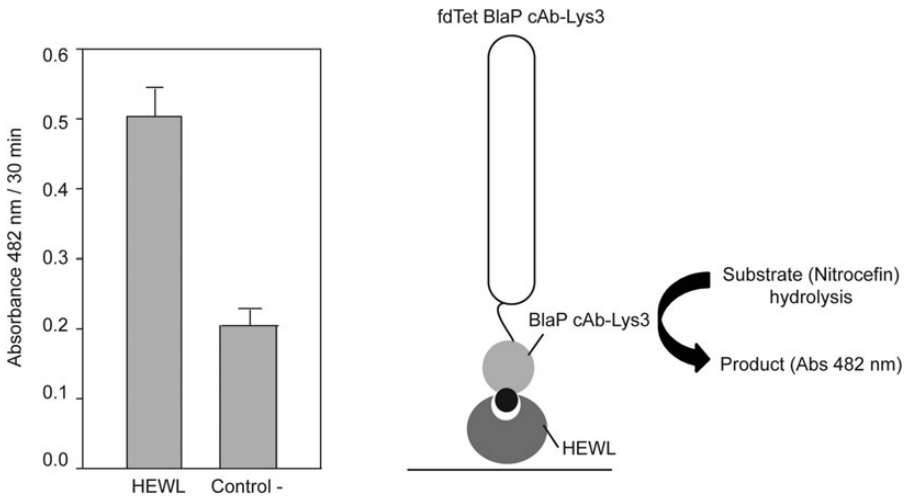

Fig. 5 Phage ELISA showing the specific interaction of BlaP cAb-Lys3 to HEWL. Phage particles displaying BlaP cAb-Lys3 on their surface were added to wells coated with HEWL or a negative control performed with an irrelevant protein coated on the wells. Unbound phages were removed by successive washings with TTBS. The bars represent the measured absorbance of the nitrocefin hydrolysis product at 482 nm. These values are directly proportional to the immobilized β-lactamase activity. The experiment was performed in triplicate.

the literature, $GlcNAc^3$ is a promising medication for human lysozyme amyloidosis therapy by preventing lysozyme misfolding and aggregation (Kumar *et al.*, 2009).

Altogether, these data demonstrate that the hybrid protein technology could be used in competition binding assays as well as drug screening.

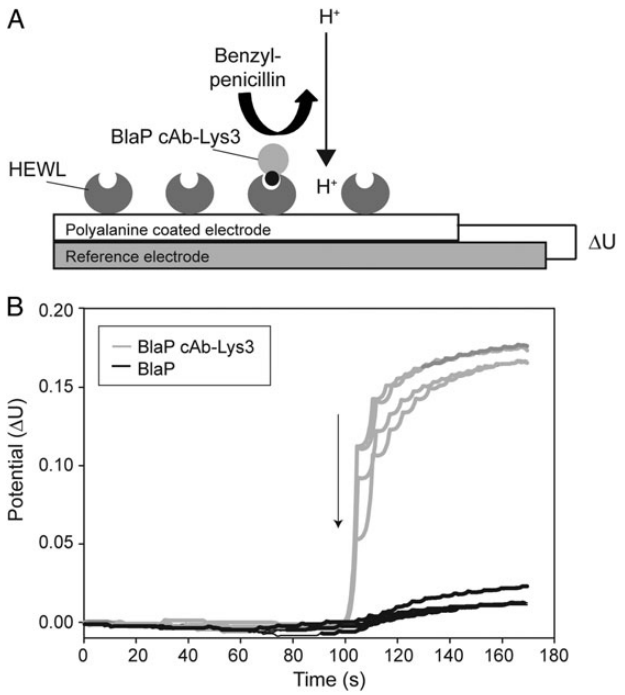

Fig. 6 Potentiometric biosensor assay showing the specific interaction of BlaP cAb-Lys3 to HEWL. (**A**) Schematic representation of the experimental setup used for the assay, where HEWL is immobilized onto a PANI (polyalanine) electrode. The hybrid protein BlaP cAb-Lys3 was then applied onto the electrode. The immobilized β-lactamase activity was determined by measuring the release of protons induced by the hydrolysis of benzylpenicillin. (**B**) Evolution of the potential difference (ΔU) observed between the PANI coated and the reference electrode in function of time upon addition of benzylpenicillin indicated by an arrow. This potential difference is induced by the proton release occurring upon antibiotic hydrolysis. The measurements were performed with the hybrid protein BlaP cAb-Lys3 (grey) and the parental β-lactamase BlaP (black), as a negative control.

Discussion

The major goal of this study consisted in proposing an innovative protein insertion technology as an alternative for functionalization of antibody fragments. Here, we reported the use of the β-lactamase BlaP as a carrier protein for displaying a camel single-domain antibody fragment (called VHH or nanobody) into a solvent-exposed loop which is diametrically opposed to the active site.

The insertion technology used in this study presents several advantages compared with the classical end-to-end fusion approach. First, insertion allows minimizing the internal flexibility of the resulting chimeric protein and therefore can improve significantly its crystallization properties (Engel *et al.*, 2002). This reduced flexibility also increases the stability of the hybrid protein against proteolysis (Collinet *et al.*, 2000). Finally, domain insertion into a carrier protein (including the class A β-lactamase TEM-1) was shown to generate hybrid protein exhibiting allosteric switch-like behavior (Ostermeier, 2005; Mathonet *et al.*, 2006).

We have chosen the β-lactamase as the carrier protein for several reasons; the activity of β-lactamases is easy to measure and quantify, these enzymes often exhibit very high turn-over numbers and many of them are quite stable. For these reasons, they are now considered as well-established reporter enzymes to study gene expression or analyze protein–ligand interactions. They are also tolerant to the insertion of relatively large proteins, without alteration of their enzymatic activity. In this study, we have generated a second generation tool and implemented it in various immunoassays that are of critical importance in both research and clinical applications. In this section, we discuss the advantages of using our protein insertion technology in the context of these assays.

Immunoassays are widely used for the detection and/or quantification of small organic analytes (e.g. drugs, hormones) or for the recognition of microbial antigens in clinical specimens. These assays involve the use of enzyme-catalyzed reactions to measure the binding of antigens to specific antibodies. Therefore, the performances of these assays strongly depend on the reaction kinetics of the enzyme–substrate system used to create a signal. Enzymes that are usually used for this purpose include alkaline phosphatases, horseradish peroxidases (Avrameas and Ternynck, 1971), β-galactosidases (Imagawa *et al.*, 1981) and β-lactamases. In this context, β-lactamases present an important advantage: these enzymes present favorable reaction kinetics which makes the assay highly sensitive and specific. Indeed, the sensitivity of β-lactamase immunoassays has been compared with that of analogous systems using alkaline phosphatase or horseradish peroxidase and was shown to be more sensitive and much easier to implement experimentally (Yolken *et al.*, 1984).

For example, β-lactamase ELISAs for various reproductive hormones have been developed and are now considered as useful tools in fertility-related problems. This system has also now replaced radioimmunoassays that use radioactive labels such as I^{125}-labeled hormones, which present a short half-time and can potentially be harmful. In addition, the handling and disposing of gamma ray emitters limit the useful lifetime of a kit and increase its cost, therefore hindering its general application (Shrivastav *et al.*, 1988a,b). For example, Shrivastav *et al.* have developed a β-lactamase-based immunoassay to detect prolactin in plasma at concentrations as low as 2.5 ng/ml (Shrivastav *et al.*, 1988a,b). This assay has then been used to detect a wide range of hormones and steroids in plasma such as testosterone derivatives (Rassaie *et al.*, 1992; He *et al.*, 1994; Prasad *et al.*, 2006), cortisol (Shrivastav *et al.*, 1988a), human chorionic gonadotropin (Prasad *et al.*, 2006), estradiol, lutropin, follitropin (Shrivastav *et al.*, 1988b). Furthermore, ready-to-use β-lactamase-based ELISA kits have been developed by Khatkhatay *et al.* (1993).

Other interesting β-lactamase-based immunoassays are those implemented in the biosensor technology that has been described in our study. Briefly, this technology relies on two mechanisms: (i) the catalytic transformation of a non-detectable molecule into a detectable form and (ii) the detection of molecules that modulate the enzyme activity. In particular, the potentiometric measurements rely on the detection of charges generated by the reporter enzyme activity. Most potentiometric biosensors use enzymes that catalyze the appearance or disappearance of protons for detection. Since β-lactam hydrolysis generates protons, β-lactamases have been used in several cases for the development of enzyme-based biosensors for diverse applications. For example, Sergeyeva *et al.* have developed a β-lactamase-based potentiometric biosensor for the detection of α–2 interferon using a highly sensitive and specific electrochemical biosensor based on pH-sensitive field effect transistors (Sergeyeva *et al.*, 1999). Gaudin *et al.* also used a similar β-lactamase-based biosensor immunoassay to screen penicillins residues in milk (Gaudin *et al.*, 2001).

Phage display is another relevant technology that has been approached in our study. In this technique, peptides, proteins or protein fragments are expressed on the surface of phage particles in fusion with one of the phage's coat proteins. This method relies on the physical link between the heterologous polypeptide expressed at the surface of the phage (phenotype) and the corresponding nucleotide sequence

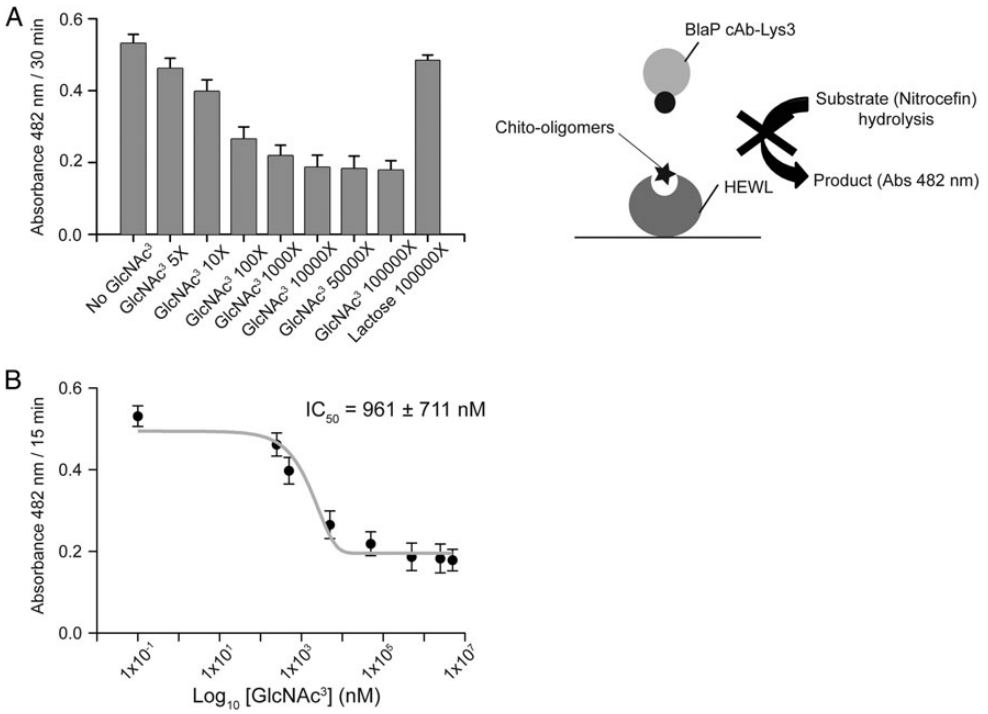

Fig. 7 Competition binding assay showing the binding of $GlcNAc^3$ and BlaP cAb-Lys3 to the active site of HEWL. This assay was performed using an ELISA experimental setup in which HEWL (5 pmol/well) was incubated with different molar excess of $GlcNAc^3$ or lactose (as a negative control). The mixtures were then coated on a microwell plate overnight at 4°C. The hybrid protein BlaP cAb-Lys3 was added to the wells and after adequate washings, the plate was incubated for 15 min with nitrocefin. The absorbance of the nitrocefin hydrolysis product was measured at 482 nm and is directly proportional to the immobilized β-lactamase activity. A control experiment was performed where no lysozyme was coated on the microplate in identical conditions compared with what has been described above. The absorbance values obtained for this control were subtracted from the corresponding values observed when lysozyme was coated on the wells. (**A**) Bar graph representing the immobilized β-lactamase activity measured for the different $GlcNAc^3$/HEWL ratios as well as for the control (lactose). (**B**) Logarithmic representation of the fitted curve used for IC_{50} determination. The data presented in panel A were fitted using the exponential (one phase decay) equation. The derived half-time (IC_{50}) and standard error calculated from three independent experiments are indicated on the graph.

cloned into the phage genome (genotype). The ability of each phage to individually replicate and expose a particular peptide on its surface allows a high-throughput screening of large libraries of variants with diversities ranging from 10^6 to 10^{10}. This technology has been extremely useful in research, in particular to study protein–ligand interaction, and medicine as well, in particular for drug discovery. However, phage display is a method that is extremely complex and technically challenging for several reasons that include (i) the lack of an easy and rapid quality control of the representativeness of the initial library, (ii) the lack of a direct assessment of the enrichment level of libraries during the successive selection rounds without interfering with the phage elution step and finally (iii) the large sequencing steps required to determine the enriched variants. All these downsides make this experiment time-consuming and labor intensive. This is why strategies have been adopted to overcome these difficulties. For example, Legendre *et al.* have combined phage display with the enzymatic activity of the *E.coli* TEM-1 β-lactamase. They inserted small peptides into two loops neighboring the active site of the enzyme (Legendre *et al.*, 2002) and displayed libraries of hybrid β-lactamases on the surface of phages in order to select them for affinity toward specific targets. Allosteric regulations (inhibition or activation) of the enzyme activity were observed for some hybrid proteins upon interaction with their specific targets (Legendre *et al.*, 1999). This coupling between the phage display technology and the enzymatic β-lactamase activity presents several advantages: (i) it decreases the conformational freedom of the displayed inserted protein fragment, (ii) it protects the inserted peptide from proteolytic cleavage, (iii) it allows to directly assess the interaction of the inserted fragments using the reporter β-lactamase activity, (iv) the enzymatic activity can also be used to monitor the enrichment of the libraries during the successive selection rounds and finally (v) it facilitates the determination of the best parameters of the assay as well as the optimization of the experimental procedure.

In conclusion, herein, we have presented a sample of possible applications for our protein insertion system and hope that this study has shown the high potential of our system and constitutes the starting point for the development of additional practically useful technologies.

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