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Rapid and easy development of versatile tools to study protein/ligand interactions

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The system described here allows the expression of protein fragments into a solvent-exposed loop of a carrier protein, the β-lactamase BlaP. When using *Escherichia coli* constitutive expression vectors, a positive selection of antibioresistant bacteria expressing functional hybrid β-lactamases is achieved in the presence of β-lactams making further screening of correctly folded and secreted hybrid β-lactamases easier. Protease-specific recognition sites have been engineered on both sides of the β-lactamase permissive loop in order to cleave off the exogenous protein fragment from the carrier protein by an original two-step procedure. According to our data, this approach constitutes a suitable alternative for production of difficult to express protein domains. This work demonstrates that the use of BlaP as a carrier protein does not alter the biochemical activity and the native disulphide bridge formation of the inserted chitin binding domain of the human macrophage chitotriosidase. We also report that the β-lactamase activity of the hybrid protein can be used to monitor interactions between the inserted protein fragments and its ligands and to screen neutralizing molecules.

Keywords: β-lactamase/antibodies/high through put screening/hybrid protein

Introduction

Many *Escherichia coli* expression vectors are available to produce and to purify proteins fused to affinity tags, such as the *Schistosoma japonicum* glutathione S-transferase (GST), the *E. coli* maltose binding protein, the *E. coli* thioredoxin etc (LaVallie and McCoy, 1995; Tsunoda *et al*., 2005). All these systems allow the subsequent proteolytic cleavage of the fusion protein to separate the protein of interest from the affinity tags (LaVallie and McCoy, 1995; Tsunoda *et al*., 2005). But a common feature of these systems is the limited utility of the affinity tag in the biochemical characterisation of the studied protein. This work describes new opportunities by using the *Bacillus licheniformis* β-lactamase BlaP as a protein carrier and a reporter enzyme.

BlaP is an active-serine enzyme belonging to class A of β-lactamases (Matagne *et al*., 1999). Class A β-lactamases share a similar three-dimensional structure characterised by all α and α/β domains (Moews *et al*., 1990; Fonzé *et al*., 1995, 2002). This enzyme is a natural proteases-insensitive protein adapted to the presence of various and numerous proteases that are co-expressed by *B. licheniformis* (Filée *et al*., 2002; Brans *et al*., 2004; Veith *et al*., 2004). Previously, we identified a common permissive insertion site in class A β-lactamases that is located in a solvent-exposed loop far away from the active site. This position limits interferences between the biological activities of the inserted polypeptide and the carrier protein (Hallet *et al*., 1997; Ruth *et al*., 2005; Chevigné *et al*., 2007). We demonstrated that small peptides (Ruth *et al*., 2005), random protein fragments (Chevigné *et al*., 2007) and protein-binding motifs (Vandevenne *et al*., 2007) can be accommodated in this loop without losing the β-lactamase activity of the carrier protein. Moreover, we showed that these hybrid proteins can be expressed at the surface of phage fdTet in fusion with the minor coat protein pIII and selected for their affinities towards specific targets (Chevigné *et al*., 2007). This new approach for displaying polypeptides is named β-lactamase hybrid protein technology (BHP) for bifunctional hybrid proteins.

In a recent report, we described the insertion of the chitin binding domain (CHBD) of the human macrophage chitotriosidase into BlaP (Vandevenne *et al*., 2007). CHBD is a protein domain of 73 residues that promotes binding to insoluble chitin. Three disulphide bridges have been reported as essential for the chitin binding activity (Tjoelker *et al*., 2000; Ujita *et al*., 2003). The BlaPCHBD construct behaved as a soluble chimeric protein that conserves both the chitin-binding and the β-lactamase activities of the related native proteins. The biochemical and biophysical properties of this protein have been studied and these results have contributed to a better understanding of the reciprocal effects between the carrier protein and the inserted protein fragment (Vandevenne *et al*., 2007).

In this work, we have engineered the permissive loop of the β-lactamase BlaP by the introduction of two thrombin (Thb)-cleavage sites surrounding the insertion site. We demonstrate here that the hybrid protein BlaP *Thb/CHBD/Thb* remains bifunctional and that the inserted polypeptide can be separated from its carrier protein without losing its biochemical properties. As a result, this approach has facilitated the high and soluble production of this small mammalian polypeptide, usually difficult to produce in *E. coli*. A relevant application has been reported where the β-lactamase activity of the hybrid protein was used to study the interaction between CHBD and chitin. Finally, it is also shown that bifunctional hybrid β-lactamases can be used in the screening of neutralizing agents.

Methods

Construction of pNY02 and pNY03

The synthetic gene fragment encoding two contiguous thrombin-cleavage sites was obtained by annealing the complementary oligonucleotides *Thb+* (5′-CTGGTTCCGCG

TGGATCTCCCGGGTTAGTGCCACGTGGTAGC-3′) and *Thb-* (5′-GCTACCACGTGGCACTAACCCGGGAGATCC ACGCGGAACCAG-3′). In this construction, the dipeptide Pro-Glu separates the proteolytic clivage sites and permits to create a *Sma*I restriction site (CCC-GGG) in the nucleotidic sequence. The annealed oligonucleotides were successively phosphorylated using T4 polynucleotide kinase and purified on a Qiaquick™ removal kit (Qiagen). The gene fragments were finally ligated into the *Sma*I-digested and dephosphorylated pNY01 (Chevigné *et al*., 2007) to yield pNY02.

The gene fragment coding for CHBD was PCR amplified using *Pfu* DNA polymerase with primers *CHBD+* (5′-GGTAGCGGACCAGAGCTTGAAGTTCCTAAACCAG GACAGCCCTCT-3′) and *CHBD−* (5′-GTGGGGTCCATTC CAGGTACAACATTTGCAGGAGTTGCTGAACACCAGAC C-3′). The PCR products were successively purified on a GFX™ gel band purification kit (Amersham Biosciences, UK), phosphorylated using T4 polynucleotide kinase and purified again on a GFX™ gel band purification kit before to be cloned into the *Sma*I-digested and dephosphorylated pNY02 to yield pNY03.

Hybrid β-lactamase purification

To achieve production of the hybrid β-lactamases harbouring a polyhistidine tag, *E. coli* JM109 transformed with pNY02 or pNY03 were grown in Terrific Broth supplemented with 75 μg/ml spectinomycin and 10 μg/ml ampicillin at 37°C. Cells from an overnight culture (1 l) were harvested by centrifugation (9000*g* for 15 min) and resuspended 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 resuspended in 100 ml of 5 mM $MgSO_4$ 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 (pH 7.4). The periplasmic proteins were loaded on a HisTrap™ Chelating HP column (GE healthcare) equilibrated in 50 mM phosphate (pH 7.4). The column was successively washed with 2 M NaCl and 50 mM phosphate (pH 7.4) supplemented with 10 mM Imidazole. The hybrid proteins were eluted by an imidazole linear gradient (10–500 mM) in 50 mM phosphate (pH 7.4). Fractions containing the purified hybrid proteins were pooled and dialysed against phosphate-buffered saline (PBS) (50 mM phosphate, 150 mM NaCl, pH 7.4).

Quantitation of the sulphhydryl groups with DTNB

Ellman's reagent, 5,5′-Dithio-bis(2-nitrobenzoic acid), DTNB (Ellman, 1959), was used for the determination of free thiols with glutathione as a standard. BlaP Thb/SmaI/Thb was used as a negative control. All measurements were performed by incubating samples in urea 6 M during 2 h to expose non-accessible cysteines in the native structures. Proteins and DTNB ($\varepsilon = 13\,600\ M^{-1}\ cm^{-1}$) concentrations were, respectively, 5 μM and 10 mM. Hundred microlitres of protein or glutathione solutions were incubated during 5 min with 900 μl of DTNB solution. Absorbance of samples was measured at 420 nm.

Thrombin cleavage

Thrombin (Sigma, Washington DC, USA) cleavage was performed in PBS supplemented with 2.5 mM $CaCl_2$ at 20°C under overnight agitation. Five units of protease were used to digest 1 mg of protein in a final volume of 1 ml. We avoided the use of 2-mercaptoethanol when working with BlaP Thb/CHBD/Thb to prevent reduction of the native disulphide bonds in CHBD. To eliminate the N- and C-terminal fragments of the β-lactamase BlaP which remain bound by non-covalent interactions, the sample was loaded on HisTrap™ Chelating HP column. Purified CHBD was collected in the flow through.

Chitin binding assay

Identical amounts (70 pmol) of BlaP Thb/SmaI/Thb, BlaP Thb/CHBD/Thb and ChBD were incubated during 2 h with 25 mg of insoluble chitin (Sigma) in a final volume of 300 μl of 50 mM sodium phosphate buffer (pH 7.5) in the presence or in the absence of 50 mM 1,4-Dithio-DL-threitol (DTT). The solutions were centrifuged at 13 000 rpm during 10 min to separate the supernatants containing unbound proteins from the pellet containing chitin with the captured proteins. The free proteins in the supernatants were analysed by SDS–PAGE and compare with the supernatants before addition of chitin.

K_r determination

Binding assays were conducted as follows: various concentrations of the chimeric protein (25 nM to 3 μM) were incubated in the presence of 10 mg of chitin in final volume of 500 μl of 50 mM sodium phosphate buffer, 150 mM NaCl, pH 7.5 at 22°C with continual mixing. The mixtures were centrifuged at 4°C for 15 min at 13 000 rpm and the supernatant containing the free protein was collected. The free protein concentration was determined using the reporter β-lactamase activity. The amount of bound protein was calculated as the difference between the initial protein concentration and the free protein concentration after binding. The relative equilibrium association constants (K_r) was determined by the method described by Gilkes *et al*. (Gilkes *et al*., 1992) using the following equation:

$$[B] = \frac{[N_0]K_a[F]}{1 + a\,K_a[F]}.$$

Where $[B]$ is the concentration of bound ligand (moles. g chitin^{-1}), $[F]$ the concentration of free ligand (molar), $[N_0]$ the concentration of binding site in the absence of ligand, a the number of lattice units occupied by a single ligand molecule and K_a the equilibrium association constant (litres.mol^{-1}). K_a value cannot be isolated from this equation but the relative equilibrium association constant, K_r (litres.g chitin^{-1}) is defined as:

$$K_r = [N_0]K_a.$$

Electrospray ionisation mass spectrometry

Purified CHBD was concentrated on a bed of PEG 20 000 to obtain a concentration of 0.25 mg/ml. Classical concentrating systems were avoided to prevent adsorption of the protein on membranes. Aggregation was observed at CHBD

concentrations >0.25 mg/ml. Before analysing the protein by mass spectrometry, the protein was dialysed against 50 mM ammonium acetate (pH 7).

Anti-CHBD antibodies

A polyclonal anti-CHBD antiserum was obtained by immunizing one New Zealand white rabbit with four injections of purified CHBD (Centre d'Economie Rurale et d'Hormonologie de Marloie).

Kinetic studies

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 linearised form according to the Hanes transformation (Frère, 1973).

Western blot analysis

Purified proteins (500 ng) were denatured at 100°C in the presence of denaturing buffer and separated by SDS–PAGE (15%). The proteins were electroblotted onto a polyvinylidene difluoride membrane. Immunoblot analyses using polyclonal preimmune or anti-CHBD rabbit sera were carried out and rabbit antibodies were detected with ECL kit (Amersham Biosciences) by using goat horseradish peroxidase-conjugated anti-rabbit antibodies.

Seroneutralisation assays

The experiments were performed in PBS (50 mM sodium phosphate, 150 mM NaCl, pH 7.4). Identical quantities (20 ng) of proteins (BlaP Thb/SmaI/Thb and BlaP Thb/CHBD/Thb) were preincubated or not with various dilutions of preimmune or anti-CHBD sera for 1 h before to be incubated during 2 h with 10 mg of insoluble chitin (Sigma). All the incubations were performed at room temperature. The solutions were centrifuged at 13 000*g* during 10 min to separate the supernatant containing unbound proteins from the pellet containing chitin with the captured proteins. The pellets were washed three times with 1 ml of wash buffer (50 mM phosphate, 500 mM NaCl, pH 7.4) and then incubated in the presence of 1 ml of 100 μM nitrocefin a chromogenic β-lactam at 25°C for 30 min. Then the suspensions were rapidly filtered through a 0.22 μm cut-off filter and the absorbances of the hydrolysis products were measured at 482 nm to determine the chitin immobilised β-lactamase activity.

Interaction of phage particles with chitin

Production and purification of phage particles harbouring the bifunctional hybrid protein or the parental β-lactamase were performed as described in our previous work (Chevigné *et al*., 2007). Interaction with phage particles was performed as follow: 100 μl of the phage particles solution ($DO_{260nm} = 5$) in 500 μl final volume of 50 mM Tris buffer (pH 7.5) were incubated 2 h with 10 mg of insoluble chitin (Sigma). The solutions were centrifuged at 13 000 rpm during 10 min to separate the supernatant containing unbound protein from the pellet containing chitin with the captured protein. The pellets were washed three times with 50 mM Tris buffer, 500 mM NaCl and then incubated in the presence of 100 μM nitrocefin for 5 min at 25°C. Then the suspension was rapidly filtered, and the absorbance of the hydrolysis product was measured at 482 nm.

Detection of fungal chitin

A colony of *Candida albicans* (ATCC 10231) was inoculated into 30 ml of YPD medium containing 2% dextrose and growth at 28°C for 20 h. To detect chitin, 4.8 ml of this culture ($DO_{600\,nm} = 0.5$) was centrifuged at 8000 rpm. The pellet washed twice with 50 mM sodium phosphate buffer (pH 7.5) and then resuspended in 500 μl of phosphate buffer containing 5 pmol of BlaP or BlaPChBD. The reaction mixture was incubated for 2 h at 4°C under continuous mixing. The cell suspensions were centrifuged at 8000 rpm during 10 min to separate the supernatant containing unbound protein from the pellet containing chitin with the captured protein. The pellets were washed three times with 50 mM sodium phosphate buffer, 0.2% Tween and then incubated in the presence of 100 μM nitrocefin for 35 min at 25°C. Then the suspension was rapidly filtered, and the absorbance of the hydrolysis product was measured at 482 nm.

Results

PNY01 is an *E. coli* plasmid that allows a high constitutive periplasmic expression of the engineered class A BlaP*/SmaI* β-lactamase (Vandevenne *et al*., 2007). The gene encoding BlaP/*SmaI* was obtained by insertion of two codons (CCC-GGG), corresponding to a *Sma*I restriction site and coding for the dipepetide Pro-Gly, between residues Asp211 and Lys212 of the BlaP β-lactamase. This plasmid was further modified by insertion into the *Sma*I restriction site of a nucleotidic sequence encoding two adjacent thrombin-cleavage sites characterised each by the consensus peptidic sequence 'Leu-Val-Pro-Arg-Gly-Ser' to give pNY02 (Fig. 1A). PNY02 leads to the production of the BlaP *Thb/SmaI/Thb* β-lactamase (Fig. 1B). In this construction, the thrombin-cleaveage sites are spaced by the Pro-Gly dipeptide, allowing a re-introduction of a *Sma*I restriction site. This site was subsequently used to clone the gene fragment encoding the CHBD of the human macrophage chitotriosidase Chit1 (residues Pro398 to Asn466) to give pNY03 (Fig. 1A). This plasmid allows the production of the BlaP *Thb/CHBD/Thb* β-lactamase. In these experiments, all the bacteria transformed with pNY01, pNY02 or pNY03 were selected and grown in the presence of 10 μg/ml ampicillin. In this case, the growth of cells results from the production in the periplasmic space of a correctly folded, functional and soluble β-lactamase. The BlaP *Thb/SmaI/Thb* and BlaP *Thr/CHBD/Thr* β-lactamases were overproduced in *E. coli* and the periplasmic extracts from overnight cultures were loaded on a HisTrap™ Chelating HP column. Thanks to the presence of a polyhistidine tag at the C-terminal end of BlaP, the two proteins were purified to homogeneity (Fig. 1C). After purification, 40 mg of BlaP *Thb/SmaI/Thb* and 18 mg of BlaP *Thb/CHBD/Thb* were obtained per litre of culture. In comparison, no cytoplasmic and periplasmic productions were obtained when we tried to express the isolated CHBD with the pET expression systems (pET 26b and pET 28a) in *E. coli* BL21 DE3 derivatives strains [BL21 (DE3) 69 450-3; BL21 (DE3) pLysS 69 451-3; Origami™ 2 (DE3) 71 345-3; Rosetta™

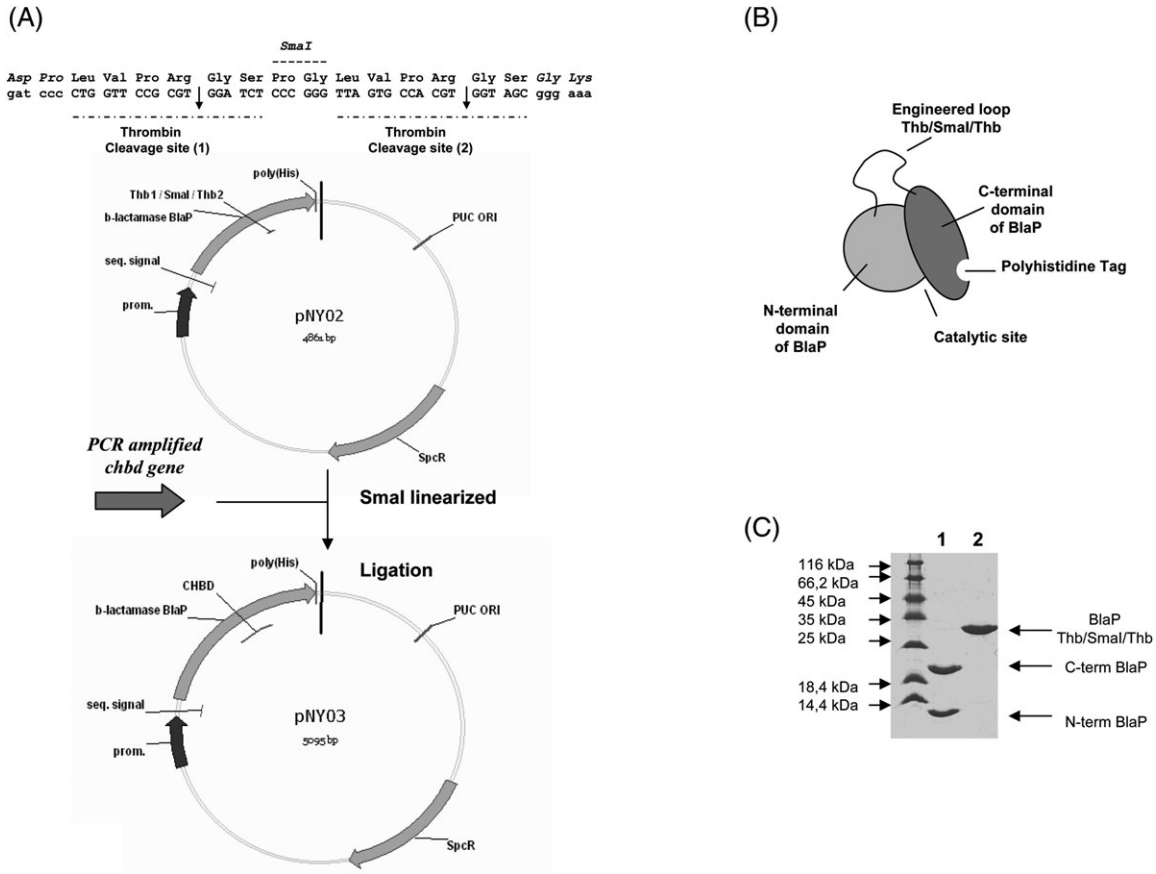

Fig. 1. Hybrid β-lactamase expression systems. (**A**) Construction of the expression vectors pNY02 and pNY03; (**B**) schematic representation of BlaP Thb/SmaI/Thb; (**C**) SDS–PAGE analysis showing a Thrombin cleavage assay performed on purified BlaP Thb/SmaI/Thb. Lane 1 corresponds to the protein molecular weight marker (Fermentas). Lane 2 corresponds to BlaP Thb/SmaI/Thb submitted to an overnight thrombin cleavage. Lane 3 corresponds to BlaP Thb/SmaI/Thb. The arrows indicate the thrombin-cleavage sites.

(DE3) 70 954-3] at different temperatures (18, 28 and 37°C). In previous studies, CHBD was poorly expressed in *E. coli* as a fusion protein with the GST (Ujita *et al*., 2003).

It has to be noticed that in our previous report (Vandevenne *et al*., 2007), we pointed out a difference (183 Da) between the theoretical and experimental molecular weights of the chimeric protein. The nucleotide sequence was verified and we did not find any mutation in the sequence encoding the hybrid protein. To explain the observed difference we also controlled the sequence of the seven-histidine tag in which we found a substitution of the first histidine to a proline and a deletion of the second one resulting only on a five-histidine tag. But fortunately, this probable recombination event did not perturb the purification process.

As shown by Table I, no free thiol was detected with BlaP *Thb/CHBD/Thb* by using Ellman's reagent in denaturing condition (6 M urea). This suggests that the six CHBD cysteines of BlaP *Thb/CHBD/Thb* are involved in the formation of three disulphide bounds. The same result was obtained with purified CHBD. The literature indicates that these disulphide bounds are of crucial importance for the correct folding and thus the chitin binding activity of CHBD (Tjoelker *et al*., 2000). The SDS–PAGE presented in Fig. 2 are in agreement with these results because the chitin binding activity of BlaP *Thb/CHBD/Thb* is completely inactivated in the presence of the reducing agent DTT.

Table I. Quantification of free thiols in BlaP Thb/CHBD/Thb and CHBD

Protein	[conc] (μM)	$[SH]_{free}$ (μM)	$[SH]_{free}$/ [Protein]	$[SH]_{th}$/ [Protein]
Glutathione	1.5	1.45	0.96	1
Glutathione	5	4.99	0.99	1
Glutathione	10	10.04	1	1
Glutathione	15	14.32	0.95	1
Glutathione	20	20.07	102	1
Glutathione	25	25.07	1	1
Glutathione	30	29.14	0.97	1
BlaP Thb-CHBD-Thb	5	0.05	0.01	6
BlaP Thb/SmaI/Thb	5	0.13	0.03	0
CHBD	5	0.07	0.01	6

DTNB was used for determination of free thiols in denaturing condition (6 M urea). Each protein was 5 μM. According to the sequences, the six cysteins are located in CHBD. Consequently, the total concentration of free SH groups is 30 μM. Standard solutions of glutathione were used to calculated the percentage of free thiols. The ratios [SH]free/[Protein] and [SH]th/[Protein] represent, respectively, the experimental and theorical number of free cysteins. The first one is determined based on the absorbance of DTNB solutions incubated with the proteins. The second one is determined based on the amino acids sequence.

BlaP *Thb/SmaI/Thb* was used as a negative control to demonstrate the specificity of the interaction between BlaP *Thb/CHBD/Thb* and chitin.

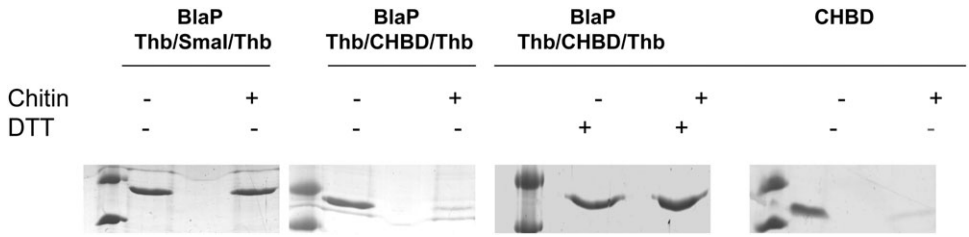

Fig. 2. SDS–PAGE analysis showing chitin binding assays performed with purified BlaP Thb/SmaI/Thb, BlaP Thb/CHBD/Thb and CHBD. The proteins were incubated in the presence of chitin. The free proteins in supernatants were analysed by SDS–PAGE. (−) corresponds to the supernatants before the addition of chitin and (+) corresponds to the supernatants after 2 h of incubation in the presence of chitin. To study the implication of disulphide bridges in the chitin binding properties of CHBD, BlaP Thb/CHBD/Thb was preincubated in the presence of 50 mM DTT before the chitin binding assay.

Table II. Kinetic studies of hybrid β-lactamases

Protein	K_m(μM)	k_{cat}(s^{-1})	k_{cat}/K_m(μM^{-1} s^{-1})
Blap WT (37°C)	35	1800	51
BlaP SmaI	48 ± 1	1260 ± 140	26 ± 2
BlaPThb/SmaI/Thb	21 ± 4	200 ± 10	10 ± 2
BlapThb/SmaI/Thb	49 ± 4	620 ± 20	13 ± 1
Thrombin-cleaved BlaPThb/CHBD/Thb	13.5 ± 2.5	235 ± 10	17 ± 1

BlaP SmaI, BlaP Thb/SmaI/Thb, BlaP Thb/CHBD/Thb correspond to the engineered forms of BlaP. The substrate was nitrocefin. The values are compared with those reported for the wild-type enzyme BlaP WT (Matagne *et al*., 1990).

The K_m and k_{cat} values of the two purified hybrid β-lactamases were compared with those of the native and BlaP/SmaI enzymes (Table II). In regard to BlaP WT, it can be concluded that the main impacts of the loop engineering are 8- and 9-fold decreases of the k_{cat} for BlaP *Thb/CHBD/Thb* and BlaP *Thb/SmaI/Thb*, respectively. But these effects can be considered as minor since they do not indicate significant structural perturbations at the level of the BlaP active site. This suggests also that the β-lactamase is correctly folded and that the CHBD inserted polypeptide is well displayed at the surface of the carrier protein. We have observed that the thrombin cleavage of BlaP *Thb/SmaI/Thb* (Fig. 1C) does not yield an inactive β-lactamase. This observation is in agreement with the data reported by Wehrman *et al*. (Wehrman *et al*., 2002). The comparison of the kinetic parameters between the cleaved and non-cleaved forms of BlaP *Thb/SmaI/Thb* (Table II) shows no significant change in the catalytic efficiency (k_{cat}/K_m) suggesting that the N-terminal and C-terminal fragments of BlaP stay in close interaction even after cleavage and that this complex is stabilised by strong non-covalent interactions.

To explain this feature, we have analysed the crystallographic three-dimensional structure of BlaP WT [PDB entry ID 4BLM (Moews *et al*., 1990)], and tried to identify the interactions which bind the N-terminal to the C-terminal parts of the protein. The Fig. 3A shows the structures of BlaP wt and its two related fragments when the protein is cleaved by thrombin. The side chains of the residues involved in the enzymatic catalysis (Ser70, Lys73, Ser130, Glu166, Asn 170 and Lys234) have been also represented. Except for Lys234, they are all included in the N-terminal fragment of BlaP. According to the literature, Lys234 could be involved in the stabilisation of β-lactam in the catalytic site during its hydrolysis (Jacob *et al*., 1990). Moreover, it appears that the C-terminal fragment of BlaP also participates in the formation of the cavity harbouring the catalytic site. If we refer to the structural organisation of class A β-lactamases, the C-terminal fragment of BlaP participates in the formation of the α/β domain of BlaP (Fig. 3B). This domain is composed of an anti-parallel β-sheet and two anti-parallel α-helices coming from the N- and C-extremities of BlaP. The C-terminal fragment of BlaP bears three β-strands of the β-sheet and one helix of the two anti-parallel α-helices. Figure 3C highlights two features involving the apolar side chains of the residues that compose the α/β domain. The first is that hydrophobic interactions appear to stabilised the anti-parallel α-helices. The second is that the two anti-parallel α-helices are bound to the β-sheet by a hydrophobic interface. The hydrogen bonds in the α/β domain and the β-sheet alone are shown in Fig. 3D and E, respectively. No hydrogen bond is established between the two anti-parallel α-helices and between the anti-parallel α-helices and the β-sheet. As expected, the five β-strands of the β-sheet establish an important number of stabilising hydrogen bonds. As mentioned early, three of the five β-strands come from the C-terminal fragment of BlaP. Thus, it is proposed that the two driving forces that stabilise the protein complex after thrombin cleavage are due to both hydrogen bonds and hydrophobic interactions.

The mutual affinity of the N-terminal and C-terminal fragments of BlaP after thrombin cleavage and the presence of a polyhistidine tag at the C-terminal end of BlaP constitute an ideal opportunity to prepare pure CHBD polypeptides. A two-step procedure has thus been validated (Fig. 4A) consisting in a cleavage of BlaP *Thb/CHBD/Thb* with thrombin in solution and followed by a depletion of the sample of BlaP fragments by loading on a HisTrap™ Chelating HP column. This step can be repeated until the desired level of purity is obtained (Fig. 4B). Mass spectrometry analysis of the depleted sample (Fig. 5) demonstrates the efficacy of the system. Only CHBD is detected as monomeric and aggregated forms. The monomeric form displays a molecular mass of 9072 Da which corresponds exactly to the expected mass after formation of the three disulphide bridges. The presence of the aggregates is in agreement with the fact that solubility problems arise upon concentration of CHBD domain when devoid of its carrier. In comparison, purified CHBD could not concentrated >0.25 mg/ml against >20 mg/ml of BlaP *Thb*/ChBD/*Thb* (corresponding to 4 mg/ml of ChBD). This shows that the β-lactamase carrier can increase the solubility of protein fragments. The chitin binding assay presented on

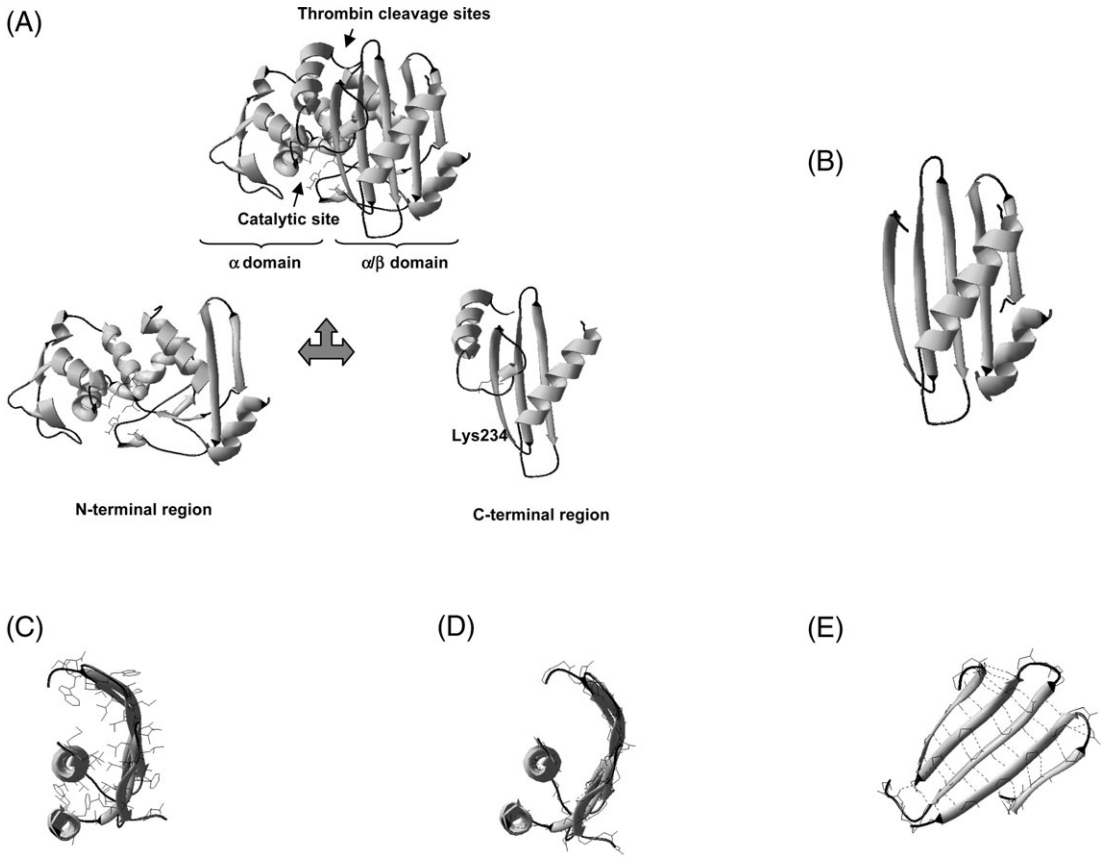

Fig. 3. Analysis of the BlaP WT crystallographic three-dimensional structure (PDB entry ID 4BLM, Moews *et al*., 1990). The lateral chain of the essential active site residues Ser 70, Lys 73, Ser 130, Glu 166, Asn 170 and Lys 234 are shown. (**A**) Structures of BlaP WT and its two fragments after thrombin cleavage. The side chains of residues directly involved in β-lactam hydrolysis are represented. (**B**) Structure of the α/β domain of BlaP WT. (**C**) Apolar side chains of residues that compose the BlaP WT α/β domain. (**D**) Hydrogen bonds in the BlaP WT α/β domain. (**E**) Hydrogen bonds in the β-sheet of the BlaP WT α/β domain.

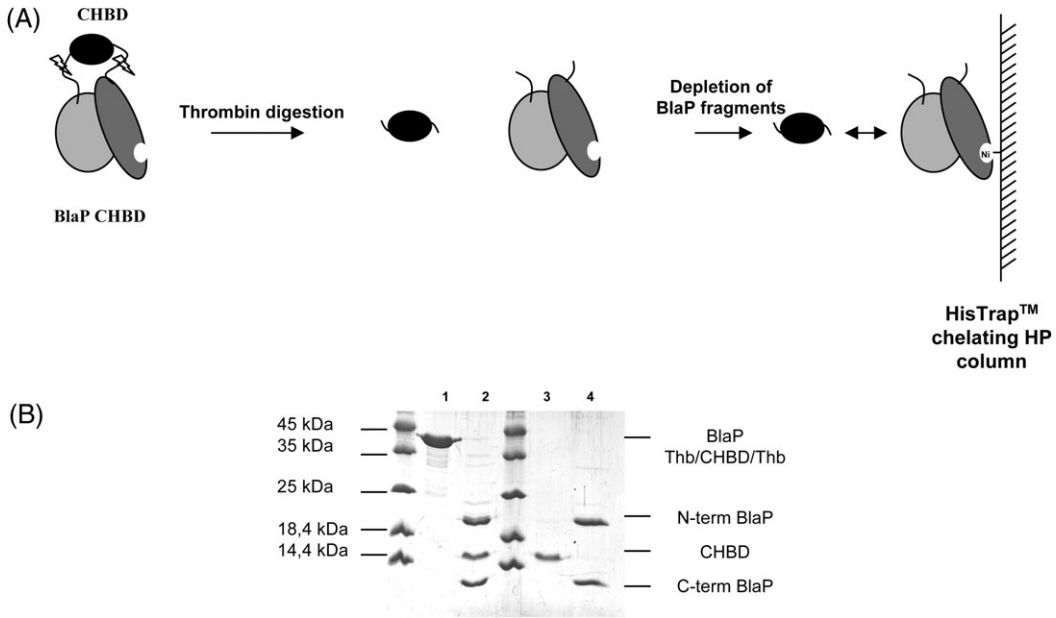

Fig. 4. Preparation of purified CHBD. (**A**) schematic representation of the protocol used to prepare purified CHBD. (**B**) SDS–PAGE analysis showing the different steps of the preparation of purified CHBD. Lane 1 and 4 corresponds to the protein molecular weight marker (Fermentas). Lane 2 corresponds to purified BlaP Thb/CHBD/Thb. Lane 3 corresponds to the thrombin-cleaved BlaP Thb/CHBD/Thb. Lane 5 corresponds to the purified CHBD collected in the flow through after loading of the sample on HisTrap™ Chelating HP. Lane 6 corresponds to the N-terminal and C-terminal fragments of BlaP Thb/CHBD/Thb eluted with imidazole from the HisTrap™ column.

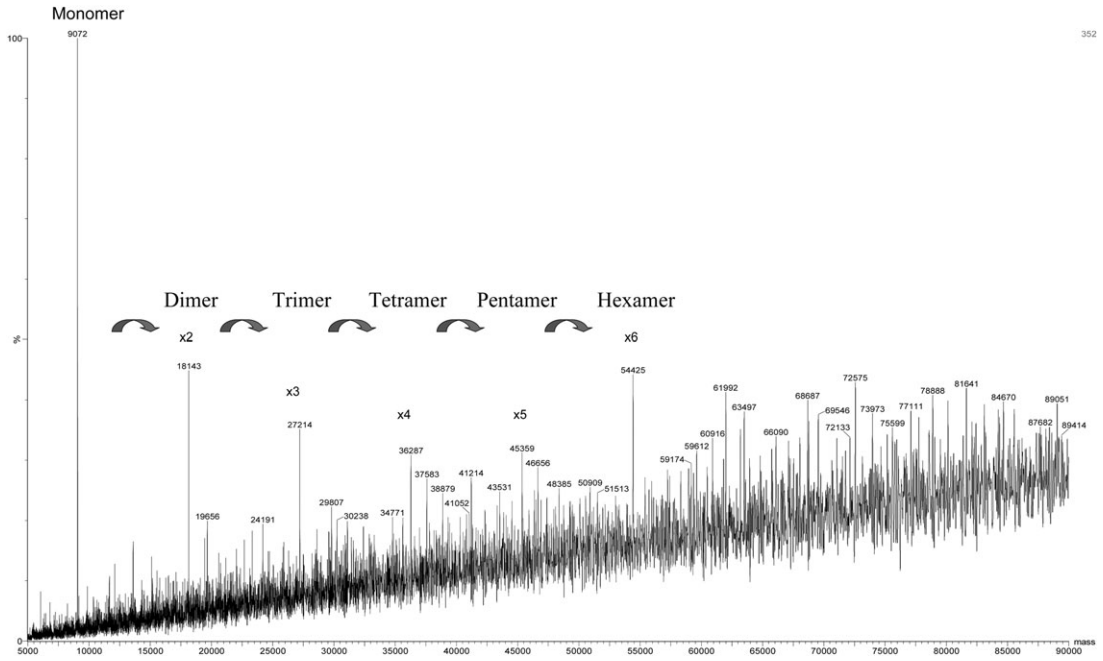

Fig. 5. Mass spectrometry analysis of the purified CHBD. The majority form is the monomer. Arrows represent the multimerisation of ChBD due to aggregation phenomenon.

Fig. 2 shows that the purified CHBD is still active. We also determined the relative equilibrium association constant (K_r) of CHBD to insoluble chitin, the calculated value is $8.5 \pm 1\,1\,g^{-1}$ which is similar to that calculated for the hybrid protein BlaPChBD namely $5.4 \pm 0.5\,l\,g^{-1}$.

Previously, we used the β-lactamase activity carried by the hybrid protein BlaP CHBD to calculate the relative equilibrium association constant (K_r) of CHBD to insoluble chitin and to quantify the amount of bound protein by measuring the free and immobilised β-lactamase activities. These β-lactamase assays were performed with nitrocefin in a colorimetric assay. In this work, we took advantage of this feature to demonstrate that the BHP offers the opportunity to screen therapeutic molecules. To validate this system, we immunised a rabbit with the purified CHBD polypeptide described above. The sera were collected after four injections. No seroneutralisation of the β-lactamase activity was observed when BlaP *Thb/CHBD/Thb* was preincubated with rabbit polyclonal preimmune and anti-CHBD sera. The western blot presented on Fig. 6 indicates that anti-CHBD antibodies have been induced after immunisation with the purified CHBD and that these antibodies recognise free CHBD, BlaP *Thb/CHBD/Thb* and the native human macrophage chitotriosidase. Figure 7 shows a seroneutralisation titration curve of the chitin binding activity of BlaP Thr/CHBD/Thr. The curve was obtained by plotting the percentage of chitin-immobilised β-lactamase activity versus the dilution of the tested sera. One hundred percent corresponds to the chitin-immobilised β-lactamase activity in the absence of the serum. These data show that immunisation with purified CHBD has induced anti-CHBD antibodies which can prevent the interaction of CHBD with chitin.

Another application of our hybrid protein system is the possibility to transpose it to phage display technology. The hybrid β-lactamase BlaPChBD can be successfully expressed at the surface of phages so that phage particles retain a

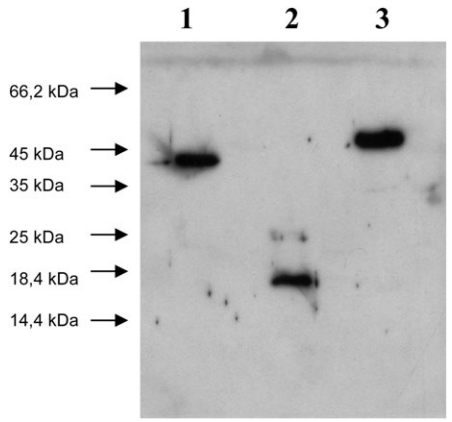

Fig. 6. Immunodetection of CHBD. The western blot was performed with rabbit anti-CHBD serum. Lanes 1, 2 and 3 were obtained with purified BlaP THb/CHBD/Thb, CHBD and human macrophage chitotriosidase.

β-lactamase activity which is used to monitor phage/ligand interactions. Figure 8 illustrates the β-lactamase activity immobilised on chitin powder and shows that this interaction is specific.

Finally, Fig. 9 shows the interaction of purified BlaPChBD with a pathogenic fungi such as *C. albicans*. The monitoring of the immobilised β-lactamase activity on this microorganism demonstrates that our hybrid protein is able to interact with chitin present in fungal cell wall and thus can be used for diagnosis fungal infections.

Discussion

During the last decade, the class A β-lactamases have emerged as promising reporter enzymes to study protein interactions. These proteins are attractive enzymes because they are monomeric and of small size (Philippon *et al*., 1998).

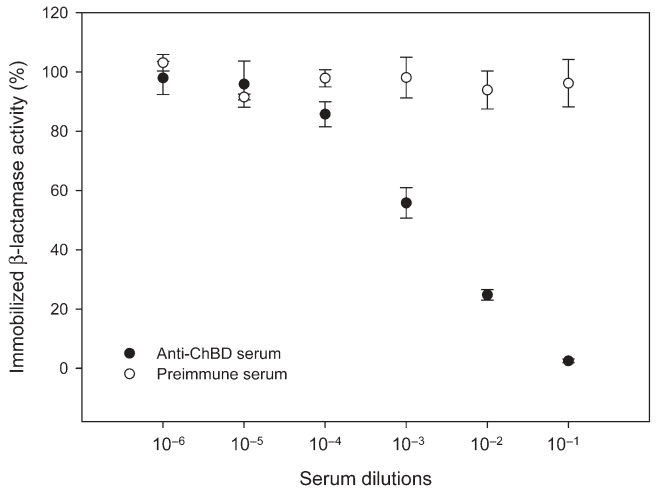

Fig. 7. Seroneutralisation curve assays of CHBD. The binding of BlaP Thb/CHBD/Thb is monitored by measuring the immobilised β-lactamase activity to chitin. Nitrocefin is used as substrate. BlaP Thb/CHBD/Thb is preincubated with increasing dilutions of serum before addition of chitin. The percentage of immobilised β-lactamase activity is plotted versus the dilution of the serum. One hundred percent corresponds to the immobilised β-lactamase activity obtained in the absence of serum.

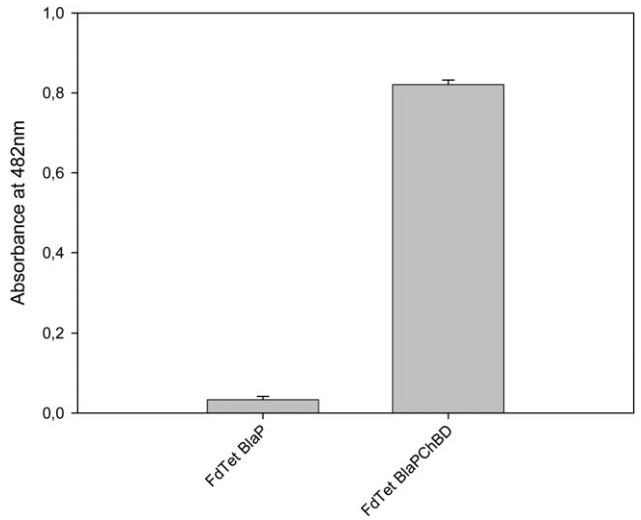

Fig. 8. Binding assay mixtures contained 10 mg (dry weight) of insoluble chitin and 100 µl of phage particle solutions ($DO_{265\,nm} = 5$) in 500 µl of 50 mM sodium Tris buffer, 150 mM NaCl (pH 7.5). Assay mixtures were incubated for 2 h at room temperature for binding. After removing the supernatant, the pellet was washed three times and then incubated for 2 min with 1 ml of nitrocefin (100 µM). After filtration of the suspension, absorbance of the nitrocefin hydrolysis product was measured at 482 nm.

Their three-dimensional structures are well-defined and they are classified as very efficient enzymes (Matagne *et al.*, 1999). β-lactamase activity is also highly specific with no equivalent in eukaryotic cells and production of β-lactamase by these cells does not result in toxic effects (Matagne *et al.*, 1998; Philippon *et al.*, 1998; Galarneau *et al.*, 2002). These proteins have been expressed successfully in prokaryotic and eukaryotic cells allowing their utilisations as well for *in vitro* as *in vivo* applications (Galarneau *et al.*, 2002). A massive expression of these enzymes can be obtained with prokaryotic expression systems allowing to drastically reduce their

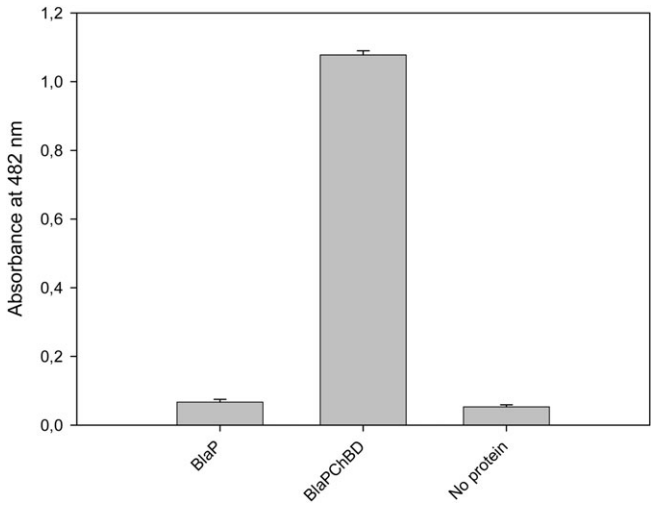

Fig. 9. Detection of fungal chitin contained in an overnight culture of *Candida albicans*. The cells are recovered by centrifugation of the culture and washed twice with 50 mM sodium phosphate buffer pH 7.5. The cell suspensions were then incubated 2 h with 25 pmol of each protein in 500 µl of 50 mM sodium phosphate buffer pH 7.5, 150 mM NaCl. After removing the supernatant, the cell pellets were washed three times and then incubated for 35 min with 1 ml of nitrocefin (100 µM). After filtration of the suspension, absorbance of the nitrocefin hydrolysis product was measured at 482 nm. A control of a suspension of *C. albicans* cells have been performed.

production times and costs (Frate *et al.*, 2000). A large variety of β-lactam substrates has been developed including chromogenic β-lactams for immunoassay, fluorescein membrane-permeant β-lactams which allow cell imagery, flow cytometry and gene expression assay, and non-toxic β-lactam prodrugs for antibody-directed enzyme prodrug therapy (Bebrone *et al.*, 2001; Vrudhula *et al.*, 2003; Lee *et al.*, 2005; Naqvi and Singh, 2007). For this latter application, a mutated β-lactamase with reduced immunogenicity has been developed (Harding *et al.*, 2005).

Many applications have been reported in the literature. For example, genetic insertion of small peptides into loops bordering the active site of β-lactamase was also used to create allosteric regulatable enzyme for homogeneous immunoassays (Legendre *et al.*, 1999). A common permissive-insertion site in class A β-lactamase has been used to insert random gene fragments. When combined with phage display, this modified enzyme has been used for epitope mapping (Chevigné *et al.*, 2007), immunoassay development and vaccine development (Ruth *et al.*, 2005). Assays based on the complementation of β-lactamase fragments fused to interacting polypeptides that regenerate enzymatic activity were used to monitor inducible protein–protein interactions in eukaryotic cells (Galarneau *et al.*, 2002; Wehrman *et al.*, 2002; Qureshi, 2007). The hydrolysis of β-lactams generates protons so that the β-lactamases are employed for the development of potentiometric signal transduction in biosensors (article submitted for publication). These examples show that β-lactamases have been successfully utilised in a wide range of applications.

This work contributes to enlarge the application fields of β-lactamase by proposing an original tool that elicits the production of difficult to express protein fragments and facilitates their purifications and studies. In a first step, this approach delivers purified hybrid proteins in which the

protein fragments of interest are each displayed into a reporter enzyme, BlaP. Thanks to the presence of the carrier protein, the binding properties of the protein fragments can be studied by using the β-lactamase activity. To allow the study of the properties of the insert independently of the presence of the carrier, two specific protease-recognition sites surrounding the insert have been introduced into the hybrid protein. By combining specific proteolytic cleavage and affinity chromatography, a purified insert was isolated. No residual trace of the carrier was detected by mass spectrometry after purification. We have demonstrated that the purified insert deleted of its carrier protein retained its biological properties. Moreover we showed that the use of the β-lactamase carrier system can be successfully used to enhance peptide expression in *E. coli* but also to increase protein solubility during sample concentration.

In a second step, we have showed that the hybrid β-lactamase is an efficient tool in the screening of molecules which can neutralise the biological properties of the studied protein fragment. In this work, the purified insert was used to immunise a rabbit and the collected polyclonal serum was shown to inhibit the binding properties of the insert by monitoring the binding of the hybrid protein to its specific ligand. This was done by measuring the immobilised β-lactamase activity. This approach can be extended to drug discovery of molecule enable to prevent or to elicit specific interactions.

We also showed that this system can be used to detect fungal infections and also easily transposed to phage display technology. This approach can be applied to the screening of enzyme-coupled protein binding domains which can also be overexpressed as recombinant proteins and directly implemented in immunoassays. This method is advantageous for affinity maturation of protein domains because the β-lactamase activity of phage particles allows the monitoring of the affinity maturation during the enrichments steps or the comparison of affinities of individual mutants without the necessity to produce a purified recombinant protein.

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