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Triazinic dye ligand selection by surface plasmon resonance for recombinant lactoferricin purification

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ABSTRACT

Bovine lactoferricin (Lfcin B) belongs to the antimicrobial peptide family, which is the first line of defense against pathogens in many organisms. Lfcin B has important applications due to its antiviral, antifungal, antiparasitic, anticancer/tumor and antibacterial activity.

In this work, we tested five triazine dyes for Lfcin B affinity interactions using surface plasmon resonance (SPR) technology. Recombinant Lfcin B was expressed as a fusion protein with GST (Lfcin B-GST) by using the baculovirus expression vector system and the dye-Sepharose matrices were assayed for Lfcin B-GST adsorption and subsequent elution.

Red HE-3B and Yellow HE-4R dyes were selected and immobilized on a Sepharose-4B matrix for further purification studies. The Yellow HE-4R-Sepharose matrix was specific for Lfcin B and allowed adsorption of Lfcin B-GST directly from the culture medium even at high salt concentration.

This novel application of SPR to screen possible dye–peptide interactions could be relevant to purify other peptides or proteins by using low-cost dye-affinity chromatography.

1. Introduction

Antimicrobial peptides (AMPs) are the first line of defense against pathogens in many organisms. AMPs are amphipathic biomolecules containing 10–100 amino acids enriched in basic residues, which provide them with a positive net charge [1]. They have antiviral, antifungal, antiparasitic, anticancer/tumor, insecticidal and/or antibacterial activity [2–8]. Around 2000 AMPs have been isolated and characterized from different sources such as microorganisms, invertebrates, plants and mammals [9]. In the last decades, many of them have been expressed by using recombinant DNA technology [10]. Different strategies, such as expression of the peptide alone [11] or in tandem [12], have been used to accomplish this purpose. The expression of a peptide alone has several disadvantages, including difficult detection, proteolytic degradation and sometimes toxicity toward the host.

Bovine lactoferricin (Lfcin B) belongs to the AMP family. Lfcin B has 25 amino acids with a net charge of +8 at physiological conditions. It is present in the N-terminal region of bovine lactoferrin (Lf B), a 80 kDa iron-binding glycoprotein found predominantly in the secreted fluids of mammals such as milk, tears, saliva, bronchial mucus and seminal plasma [13]. Lfcin B comprises the residues 17–41 of Lf B and is released by acidic pepsin hydrolysis. Besides having all the functions of AMPs, Lfcin B has a synergistic effect with some conventional antifungal, antiviral and antibacterial drugs [14–16]. Lfcin B has been recombinantly expressed in various expression systems using different strategies for its recovery and purification [17–22]. In many cases, Lfcin B is expressed in tandem as multimers [19] or as a fusion protein with glutathione-S-transferase (GST) or thioredoxin to avoid Lfcin B toxicity toward the host and to minimize proteolytic degradation [17,18,21]. When Lfcin B is expressed in tandem as multimers, the downstream processing usually involves cell lysis, clarification, concentration and conditioning of the sample previous to ion exchange chromatography. Then, a chemical agent like cyanogen bromide, formic acid or hydroxylamine is necessary to release the peptide. These reagents can cause side-chain amino acid modification [10]. When Lfcin B is expressed with GST, the fusion protein is purified using GST affinity chromatography after concentration and conditioning of the sample. This last approach has some advantages, because the fusion protein masks the toxicity of the peptide, avoids proteolytic degradation and allows the detection by antibodies or by measuring GST enzymatic activity. Then, a specific proteolytic enzyme, like

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bovine thrombin, is used to separate Lfcin B from GST, followed by size exclusion chromatography or ultrafiltration to separate Lfcin B from other proteins.

Triazine dyes have been extensively used as ligands to obtain affinity chromatography matrices owing to their low cost, ready availability, simple immobilization reaction, resistance to biological and chemical degradation, and acceptable capacity and selectivity [23]. These advantages make them highly selective for large-scale purification processes [24]. Traditionally, the selection of a triazine dye as an affinity ligand for a specific protein is an empirical and time-consuming process that involves the screening of a number of different dyes, the immobilization of each on a matrix and the evaluation of their selectivity against the target protein [23,25–28]. Lf B has been previously purified from bovine whey and colostrum by using immobilized triazine dyes as ligands in different supports [28]. The triazine dye selected after traditional screening was Red HE-3B.

Surface plasmon resonance (SPR) is a powerful technology which has become a widely used tool to study protein–protein interactions without labeling requirements [29]. SPR detects changes in the refractive index when a specific interaction occurs on a sensor surface, providing different kinetic and equilibrium parameters [30].

Based on previous results [25,27,28], in this work, we tested five triazine dyes -Red HE-3B, Reactive Green 19, Yellow HE-4R, Cibacron Blue F3GA and Red F5B- for Lfcin B affinity interactions using SPR.

2. Materials and methods

2.1. Analytical reagents

The molecular weight standards PageRuler™ Prestained (Thermo Scientific, Rockford, IL, USA) and BenchMark™ Protein Ladder (Invitrogen, Gaithersburg, MD, USA) were used for Western blot. The PD-10 desalting columns, nitrocellulose membrane and reagents for chemiluminescence detection were from GE Healthcare (Piscataway, NJ, USA). The Quick start™ Bradford reagent for total protein determination was obtained from BioRad (Hercules, CA, USA). Cellfectin reagent was from Invitrogen and 1-chloro-2,4-dinitrobenzene, 3,3′-diaminobenzidine, Sepharose 4B, Red HE-3B dye, Cibacron Blue F3GA dye and Reactive Green 19 dye were from Sigma–Aldrich (St. Louis, MO, USA). Yellow HE-4R dye and Red F5B dye were from Vilmax S.A. (Buenos Aires, Argentina). Centrifugal filters with a cut-off of 10 kDa were from Millipore (Massachusetts, USA). The mouse anti-GST primary monoclonal antibody was obtained from BD Biosciences (San Diego, CA, USA). The rabbit anti-mouse Horseradish peroxidase (HRP)-conjugated secondary antibody was obtained from DAKO (Glostrup, Denmark). Synthetic Lfcin B used as a standard was from GenScript (Piscataway, NJ, USA).

2.2. Molecular biology

The DNA sequence of Lfcin B was synthesized and cloned in the pUC18 vector by GenScript. Transfer vectors (pAcSecG2 T and pAcGP67B) and Baculogold Bright AcMNPV DNA were from BD Biosciences. The insect cell line IPBL-Sf9 from *Spodoptera frugiperda* (*Sf*9) was purchased from the Asociación Banco Argentino de Células (ABAC, Pergamino, Buenos Aires, Argentina). Sf900 II insect culture media and the antibiotic and antimycotic solution were from Invitrogen (Carlsbad, CA, USA). The fetal calf serum was from Nutrientes Naturales S.A. (Buenos Aires, Argentina).

2.3. Recombinant baculovirus constructions

The construction named pAcGST-Lfcin B containing the DNA sequence of Lfcin B fused to the GST gene (from *Schistosoma japonicum*), preceded by an in-frame gp67 signal sequence to allow secretion of the fusion protein into the supernatant was cotransfected with Baculogold Bright AcMNPV DNA according to BD Biosciences protocols.

In addition, two baculoviruses were constructed for control experiments. The pAcSecG2T transfer vector was used to construct a recombinant baculovirus (BacGST) using the same protocol. BacGST was used to express GST in culture medium. To construct the baculovirus BacLfcin B, the DNA sequence of Lfcin B was cloned into transfer vector pAcGP67B, Cells infected with this recombinant baculovirus (BacLfcin B) express and secrete Lfcin B in the culture medium. This baculovirus was used to determine the basal GST enzymatic activity during infection.

2.4. Expression of Lfcin B-GST in insect cell cultures

Sf9 suspension cultures (2×10^6 cells/ml) grown in Sf900 II medium supplemented with 1% of fetal bovine serum were infected with BacGST-Lfcin B or BacGST at a multiplicity of infection (MOI) of 0.5. Samples of 1 ml were collected each day post-infection to determine GST enzymatic activity and for protein analysis by Western blot. Briefly, the culture medium was separated from the cells by centrifugation at $10{,}000 \times g$ for 10 min. The pellet was discarded and the supernatant stored at −20 °C until further experiments.

2.5. Western blot analysis

Samples from supernatants were quantified by the Bradford assay [31] using the Quick start™ Bradford reagent, and 10 μg of protein from each day post-infection was loaded and separated in a 15% SDS-PAGE. Proteins were blotted onto a nitrocellulose membrane. The membrane was blocked with 3% skim milk in phosphate buffer solution (PBS), pH 7.0, for 1 h at room temperature, and then incubated with anti-GST primary antibody (against GST from *S. japonicum*) for 1 h at room temperature (1:1000 diluted in PBS with 0.3% non-fat milk). After three washes of 15 min each with PBS-0.05% Tween 20, the membrane was incubated with an HRP-conjugated secondary antibody (1:1500), washed again and revealed by chemiluminescence or using 3,3′-diaminobenzidine.

2.6. Determination of GST enzymatic activity

The conjugation of glutathione with 1-chloro-2,4-dinitrobenzene (CDNB) by GST was monitored at 340 nm according to Zhang method [32]. The amount of Lfcin B-GST was calculated from the mass ratio compared to the amount of a standard curve of GST activity determined using a GST standard from GenScript.

2.7. Surface plasmon resonance (SPR) assay

The affinity of the soluble triazine dyes tested -Red HE-3B, Reactive Green 19, Yellow HE-4R, Cibacron Blue F3GA and Red F5B- with immobilized Lfcin B was determined by SPR analysis, using a Biacore T100 instrument (Biacore Inc., Piscataway, NJ, USA). The synthetic Lfcin B (~100 μg/ml) was dialyzed against 10 mM sodium acetate, pH 4.0, and coupled to the carboxymethyl-dextran matrix of CM5 sensor chips (Biacore) using the Amine Coupling Kit, according to Johnsson et al. [33]. Briefly, the CM 5 sensor chip surface was activated by 35 μl injection of NHS/EDC mix, transforming carboxymethyl goups to N-hydroxysuccinimide esters. After the baseline was reached, 150 μl of ligand was injected. The N-terminus and ε-amino groups of lysine residues of Lfcin B reacted spontaneously with the N-hydroxysuccinimide esters. To quench the remaining activated sites, a saturation concentration of ethanolamine was injected. The activation and immobilization periods were set automatically with the aim to couple 1200 resonance units (RU). Two-fold dilutions of micromolar concentrations of triazine dyes in PBS, pH 7.0 were assayed over the control and reactive flow cell. All binding experiments were performed at 25 °C. Dissociation and surface regeneration were carried out in PBS. SPR data were analyzed using the BIAcore T100 evaluation software (Biacore). All the experiments were repeated at least three times and the standard deviations were typically less than 9%. Dissociation constants (K_D) were determined under equilibrium binding or kinetic conditions after correction for nonspecific binding, in which the triazine dyes were passed over blocked, empty flow cells, as previously described [34–37].

2.8. Synthesis of dye-Sepharose 4B

Red HE-3B and Yellow HE-4R were immobilized on Sepharose 4B as described by Stellwagen [38] with minor modifications. About 10 g of Sepharose 4B was suspended in 10 ml of distilled water containing 250 mg of either dye and then 5 ml of 4 M NaCl was added to the suspension. The mixture was gently stirred for 1 h at room temperature, then 500 μl of 10 M NaOH was added and the suspension was made up to a final volume of 50 ml with distilled water. After 10 h of agitation, temperature was raised to 40 °C and the mixture incubated for 4 h. Finally, each dye-Sepharose matrix was washed several times with water, methanol, 2 M NaCl and 1 M NH_4Cl to remove the excess of dye.

2.9. Batch purification

The culture medium was separated from the cells by centrifugation at $10{,}000 \times g$ for 10 min. The pellet was discarded and the supernatant was used directly with the dye-Sepharose matrices without any pretreatment. Then, 100 μl of the Red HE-3B-Sepharose matrix (R-S) or Yellow HE-4R-Sepharose matrix (Y-S), previously equilibrated in 50 mM sodium phosphate buffer pH 7.0, was added to 1 ml of culture supernatant. The suspension was mechanically stirred for 12 h at 4 °C. Then, after discarding the supernatant, matrices were washed four times with 1 ml of 50 mM sodium phosphate buffer, pH 7.0, with 0.5 M NaCl for 1 h each wash. The same process was also performed with the addition of 0.5 or 1 M NaCl to the clarified culture supernatant prior to the adsorption step.

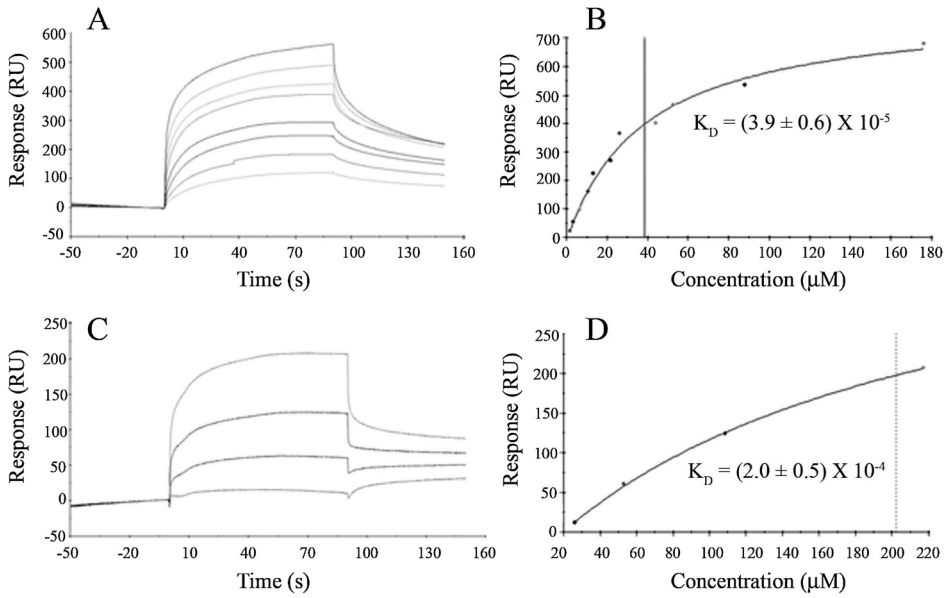

Fig. 1. Specific interaction between triazine dyes of Cluster 1 and Lfcin B. Sensorgrams showing the specific interaction between Reactive Green 19 (A) or Red F5B (C) and Lfcin B. K_D values were determined with a non-linear analysis under equilibrium conditions (Steady State Analysis) using BIAevaluation software for Reactive Green 19 (B) or Red F5B (D).

Different eluents (2 M NaCl, 2 M NaCl with 25% ethylene glycol, 0.5 M NaSCN, 0.5 M NaSCN with 25% ethylene glycol and 25% ethylene glycol) were tested overnight for the recovery of the fusion protein. Each of them was tested at different pH values: pH 7.0 (50 mM sodium phosphate buffer), pH 9.0 (50 mM Tris–HCl buffer) and pH 10.0 (50 mM carbonate buffer). Lfcin B-GST was quantified by direct determination of GST enzymatic activity. Centrifugal filters or PD-10 desalting columns were used to condition the elution samples. Protein was quantified by the Bradford assay [31] and 15 μl from each purification step was loaded and separated in a 15% SDS-PAGE for Western Blot.

3. Results and discussion

In order to select a ligand to purify Lfcin B-GST the affinity and kinetic parameters of five different triazine dyes by Lfcin B were determined by SPR, using the BIAcore T100 equipment.

3.1. SPR analysis

Synthetic Lfcin B (1200 RU) was coupled directly to the dextran matrix of a sensor chip CM5 through primary amine groups of the peptide and different concentration of dyes were passed over for 90 s. Dissociation phase was also set at 90 s and carried out with PBS, pH 7.0. The analysis of the binding profiles showed that the dyes presented different kinetic properties to bind Lfcin B, as indicated below. Since the stoichiometry of the interaction is unknown, the experimental curves were fit to a binding model 1:1, to avoid an overfitting. Based on the kinetic parameters, we grouped the triazine dye-Lfcin B interactions in three clusters: Cluster 1, integrated by Reactive Green 19 and Red F5B (Fig. 1); Cluster 2, integrated by Red HE-3B and Cibacron Blue F3GA (Fig. 2); and Cluster 3, with only one member, Yellow HE-4R (Fig. 3). Data were analyzed using

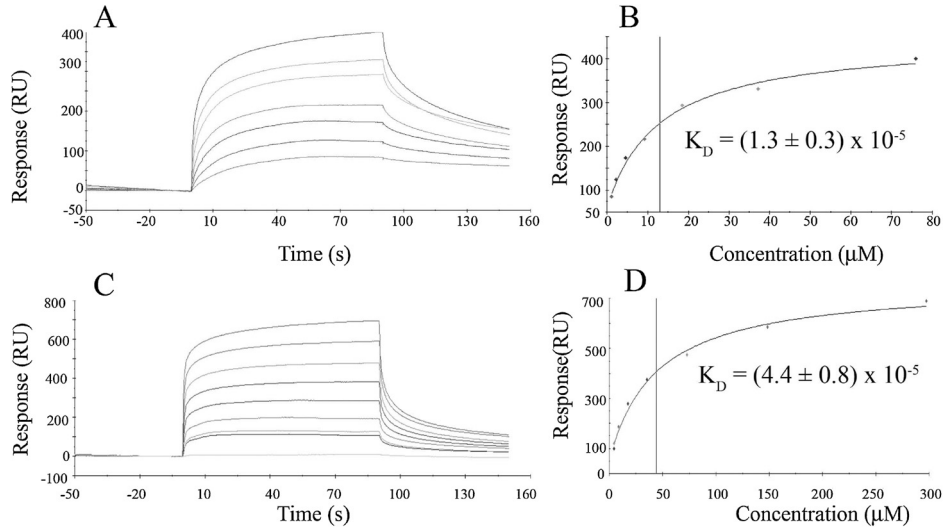

Fig. 2. Specific interaction between triazine dyes of Cluster 2 and Lfcin B. Sensorgrams showing the specific interaction between Red HE-3B (A) or Cibacron Blue F3GA (C) and Lfcin B. K_D values were determined with a non-linear analysis under equilibrium conditions (Steady State Analysis) using BIAevaluation software for Red HE-3B (B) or Cibacron Blue F3GA (D).

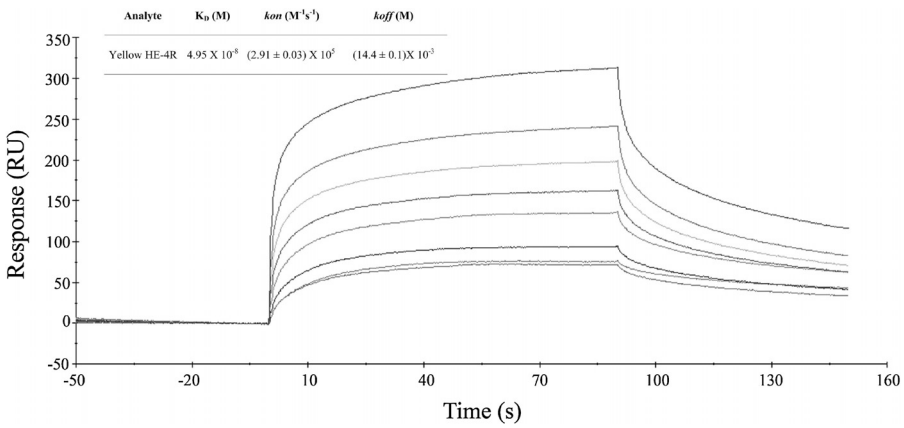

Fig. 3. Specific interaction between triazine dyes Cluster 3 and Lfcin B. Sensorgram showing the specific interaction between Yellow HE-4R with Lfcin B. K_D and association (k_{on}) and dissociation (k_{off}) rates were determined with kinetic analysis using BIAevaluation software (insert).

Table 1
Binding affinities and kinetic parameters obtained by SPR of triazine dyes for Lfcin B.

	Dye	K_D (M)	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})
Cluster 1	Reactive Green 19	$3.9. \pm 0.6 \times 10^{-5}$ [a]	ND	$(993.7 \pm 0.1) \times 10^{-5}$
	Red F5B	$2.0 \pm 0.5 \times 10^{-4}$ [a]	ND	$(217.1 \pm 0.1) \times 10^{-5}$
Cluster 2	Red HE-3B	$1.3 \pm 0.3 \times 10^{-5}$ [a]	1012.3	$(131.46 \pm 0.9) \times 10^{-4}$
	Cibacron Blue F3GA	$4.4 \pm 0.8 \times 10^{-5}$ [a]	511.6	$(225.1 \pm 0.92) \times 10^{-4}$
Cluster 3	Yellow HE-4R	4.95×10^{-8} [b]	$(2.91 \pm 0.03) \times 10^{-5}$	$(14.4 \pm 0.1) \times 10^{-3}$

[a] K_D were determined under steady-state conditions.
[b] K_D was determined under kinetic conditions.

BIAevaluation T100 software, which allows the analysis under kinetic or equilibrium conditions (Table 1). Due to the chemical nature of the dyes, it was not possible to couple them to the surface of the sensor chip in order to analyze the binding in reverse orientation.

Injection of different concentrations of the dyes from Cluster 1, Reactive Green 19 ($0.016–1.7 \times 10^{-4}$ M, Fig. 1A) and Red F5B ($0.26–2.5 \times 10^{-4}$ M, Fig. 1C), with immobilized Lfcin B gave a concentration-dependent specific binding. The binding of the two dyes to Lfcin B was characterized by a very slow association rate which was outside the limits that could be accurately measured. Consequently, affinities were determined under equilibrium binding conditions. The corresponding steady-state affinity analysis is shown in Fig. 1B for Reactive Green 19 and Fig. 1D for Red F5B. The apparent K_D values determined were $(3.9 \pm 0.6) \times 10^{-5}$ M and $(2.0 \pm 0.5) \times 10^{-4}$ M, respectively. In contrast, the values of the k_{off} could be properly determined (Table 1).

Injection of different concentrations of the dyes from Cluster 2, Red HE-3B ($0.12–7.6 \times 10^{-5}$ M, Fig. 2A) and Cibacron Blue F3GA ($0.04–3 \times 10^{-5}$ M, Fig. 2C), with immobilized Lfcin B also gave a concentration-dependent specific binding. In this cluster the binding to Lfcin B was characterized by a moderately slow association rate that could be estimated by the program, but was not completely accurate. Considering this limitation, affinities were also determined under equilibrium binding conditions. The corresponding steady-state affinity analysis is shown in Fig. 2B for Red HE-3B and Fig. 2D for Cibacron Blue F3GA. The apparent K_D values determined were $(1.3 \pm 0.3) \times 10^{-5}$ M and $(4.4 \pm 0.8) \times 10^{-5}$ M, respectively. As before, the k_{off} were properly determined (Table 1).

A similar assay conducted with Yellow HE-4R ($0.02–1.5 \times 10^{-6}$ M) allowed the accurate determination of the association and dissociation rates in a concentration-dependent specific binding. As shown in Fig. 3 and Table 1, this dye associated with Lfcin B with a k_{on} of $(2.91 \pm 0.03) \times 10^5$ M^{-1} s^{-1} and dissociated with a $k_{off} = (14.4 \pm 0.1) \times 10^{-3}$ s^{-1}. These results determined a K_D of 4.95×10^{-8} M.

In a purification process, it is desirable that the concentration of the target protein is at least in the order of the K_D for maximum adsorption to a ligand immobilized in a matrix. The K_D values were considered to select the dyes once the concentration of Lfcin B secreted as a fusion protein with GST in the culture medium using BEVS was determined.

3.2. Expression of Lfcin B-GST

Expression of Lfcin B-GST using BEVS was confirmed using enzymatic activity of GST and Western blot. Fig. 4 shows the accumulation of Lfcin B-GST in the culture medium at different times post-infection. When the cells were infected with the recombinant baculovirus BacGST-Lfcin B, the fusion protein was secreted to the culture medium. The GST activity detected in the culture medium

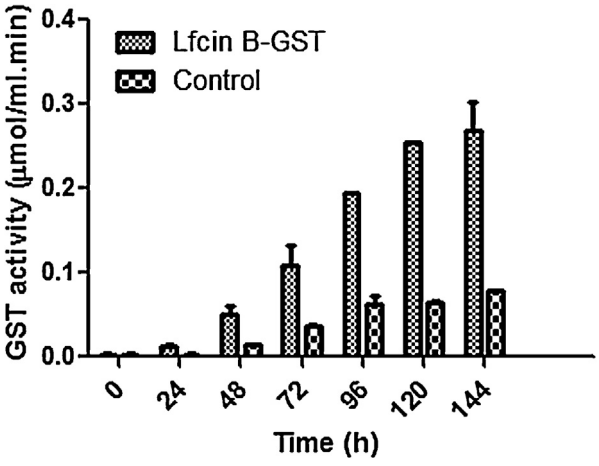

Fig. 4. Expression kinetics of Lfcin B-GST in culture medium determined by GST activity. Control bars represent the GST activity in the supernatant of cell cultures infected with the baculovirus BacLfcin B.

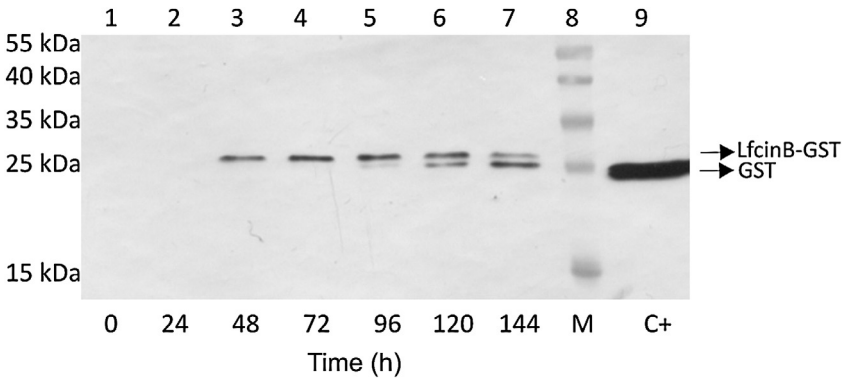

Fig. 5. Expression kinetics of Lfcin B-GST analyzed in culture medium by using Western blot. Lanes 1 to 7, expression of Lfcin B-GST at 0, 24, 48, 72, 96, 120 and 144 h after infection with the baculovirus BacGST-Lfcin B, respectively. Lane 8, protein ladder. Lane 9, expression of GST in culture medium at 96 h post-infection with the baculovirus BacGST.

increased with the course of infection. This GST activity was almost four-fold greater than the GST basal activity of the control recombinant baculovirus (BacLfcin B). The basal GST activity detected may be attributed to the GST endogenous activity produced during baculovirus infection (Fig. 4, control bars). Thus, approximately 70% of the GST activity detected in the culture medium of cells infected with BacGST-Lfcin B corresponded to the recombinant protein Lfcin B-GST. Fig. 5 shows the accumulation of Lfcin B-GST in the culture medium at different times post-infection, using Western blot detection. The anti-GST primary antibody was specific against the GST fraction of the recombinant Lfcin B-GST. At 120 h and 144 h post-infection, a double band was detected (Fig. 5, lanes 6 and 7). Typically, this may be a consequence of partial degradation of the recombinant protein as a result of generalized cell lysis and the release of proteases into the culture medium in the last stages of baculovirus infection.

For further purification experiments, the culture medium of Lfcin B-GST expression at 96 h post-infection was selected. At this time, the yield was 10 mg of Lfcin B-GST per liter (1.1 mg of Lfcin B per liter).

In previous experiments, Lfcin B was expressed without GST, using BEVS. Probably due to low yield or degradation in the culture medium, it was not possible to detect Lfcin B (data not shown). On the other hand, the use of a fusion protein as a strategy to express Lfcin B gives stability to the peptide and eases its detection by using enzymatic activity or Western blot analysis.

The K_D of Lfcin B for Cibacron Blue F3GA, Reactive Green 19 and Red F5B was in the order of 4×10^{-5}–2×10^{-4} M, according to steady-state conditions using SPR (Table 1). These values are 100 and 1000 times above the concentration of Lfcin B (expressed as Lfcin B-GST fusion protein) in the culture medium ($\sim 3.5 \times 10^{-7}$ M). Therefore, these dyes were discarded for further purification of Lfcin B-GST.

On the other hand, the K_D of Lfcin B for Yellow HE-4R was 4.95×10^{-8} M according to the kinetic parameters using SPR. Since the K_D values for Yellow HE-4R were in the order of the concentration of Lfcin B (expressed as Lfcin B-GST fusion protein) in the culture medium ($\sim 3.5 \times 10^{-7}$ M), this dye was selected for immobilization to a matrix and further purification experiments.

The K_D of Lfcin B was $1.3 \pm 0.3 \times 10^{-5}$ M for Red HE-3B, according to steady-state conditions using SPR. Grasselli et al. studied the interaction between Red HE-3B dye and Lf B using molecular modeling and suggested that Red HE-3B interacts with the N-terminal region of Lf B where Lfcin B is present [39]. Although the K_D value for Red HE-4R was 100 times above the concentration of Lfcin B in the culture medium, previous results with Lf B have shown that this dye is a possible ligand for Lfcin B and was thus also selected for further purification of Lfcin B-GST [25,27,28].

When the adsorption of pure Lfcin B with the five dyes immobilized on Sepharose 4B at a concentration similar to those obtained in the culture medium of insect cells and at lower concentrations was tested, a correlation between the K_D values obtained in SPR and the Lfcin B adsorption was observed. The K_D values for the five dyes were: 2.15×10^{-5} M for Red F5B, 3.2×10^{-5} M for Reactive Green 19, 2.4×10^{-6} M for Cibacron Blue F3GA, 4.7×10^{-6} for Red HE-3B and 5.9×10^{-7} for Yellow HE-4R. These results prove that our approach was efficient for the selection of a dye-ligand for Lfcin B.

The selected dyes were immobilized onto a Sepharose 4B matrix and used to purify Lfcin B-GST from the culture medium.

3.3. Batch adsorption using dye-Sepharose matrices

The culture medium was separated from the cells by centrifugation and used directly to purify Lfcin B-GST without any pretreatment. The pH of the culture medium was 6.4 and the conductivity 7 μS/cm. Fig. 6 shows the adsorption of Lfcin B-GST from the culture supernatant to the dye-Sepharose matrices. The Yellow HE-4R Sepharose matrix (Y-S) showed $67.7 \pm 1.8\%$ of adsorption while the Red HE-3B Sepharose matrix (R-S) showed $74.9 \pm 1.9\%$ of adsorption, both with respect to the initial GST activity before batch purification (Fig. 6A). However, when the adsorptions were analyzed by Western blot, the adsorption of Lfcin B-GST using either Y-S or R-S was around 100% because no Lfcin B-GST was detected (Fig. 6B, lanes 4 and 5).

Consequently, the adsorption of Lfcin B-GST to the selected dye-Sepharose matrices was complete and the remaining GST activity detected after the experiments of batch adsorption is intrinsic for Sf9 infected cells (Fig. 4, control bars).

The interaction between Lfcin B-GST and dye-Sepharose matrices was slightly affected by the increase in the ionic strength caused by the addition of NaCl to the culture medium, as shown in Fig. 6B and C (lanes 4–9). In the presence of 0.5 M NaCl, the adsorption of Lfcin B-GST to both dye-Sepharose matrices decreased by 10% compared to the adsorption without NaCl. With 1 M NaCl, adsorption of Lfcin B-GST decreased by 35% using Y-S and by around 15% using R-S. This reveals the existence of electrostatic interactions between Lfcin B-GST and the dye-Sepharose matrices, which are more evident with Y-S. Also, a hydrophobic component may be involved in the interaction between Lfcin B-GST and dye-Sepharose matrices. This kind of interactions may be favored by the increase in the ionic strength of the culture medium and may be responsible for the adsorption to the dye-Sepharose matrices together with the electrostatic interactions.

The results shown in Fig. 6 correlated with the results obtained using SPR technology (Table 1). To determine if the interaction of

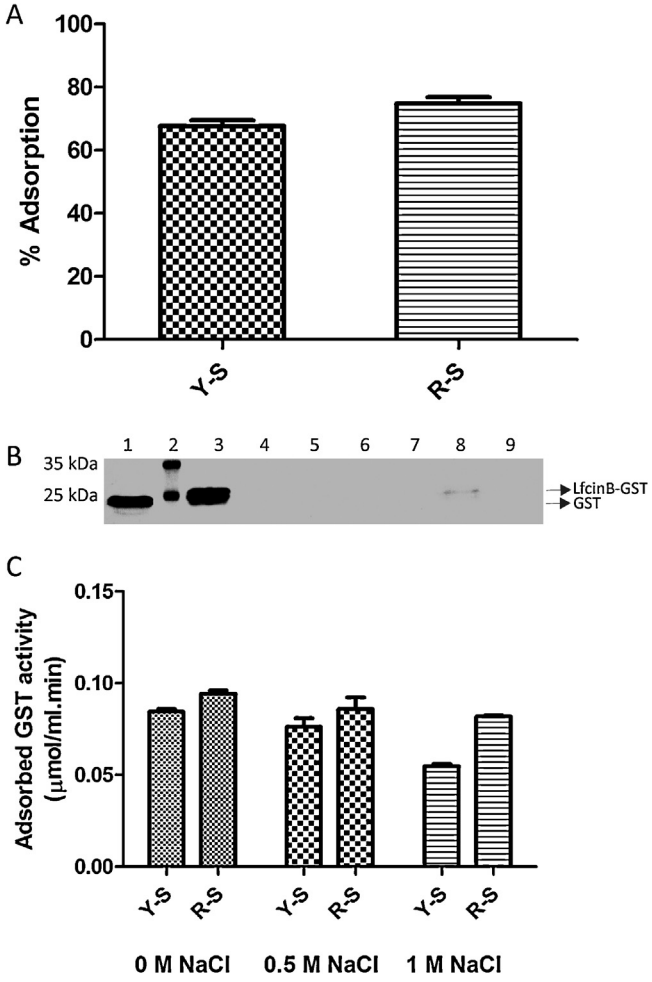

Fig. 6. Adsorption of Lfcin B-GST to dye-Sepharose matrices. (A) Adsorption of Lfcin B-GST to Yellow HE-4R-Sepharose matrix (Y-S) and to Red HE-3B-Sepharose matrix (R-S). Results are expressed as a percentage of the initial GST activity. (B) Analysis of adsorption of Lfcin B-GST to Y-S and R-S by Western blot. Lane 1, expression of GST in culture medium. Lane 2, protein ladder. Lane 3, expression of Lfcin B-GST in culture medium. Lanes 4, 6 and 8, pass-through of Y-S without NaCl, with 0.5 M NaCl and with 1 M NaCl, respectively. Lanes 5, 7 and 9 pass-through of R-S without NaCl, with 0.5 M NaCl and with 1 M NaCl, respectively. (C) Adsorption of Lfcin B-GST to Y-S and to R-S expressed in total adsorbed enzymatic GST units and performed without NaCl, 0.5 M NaCl or 1 M NaCl.

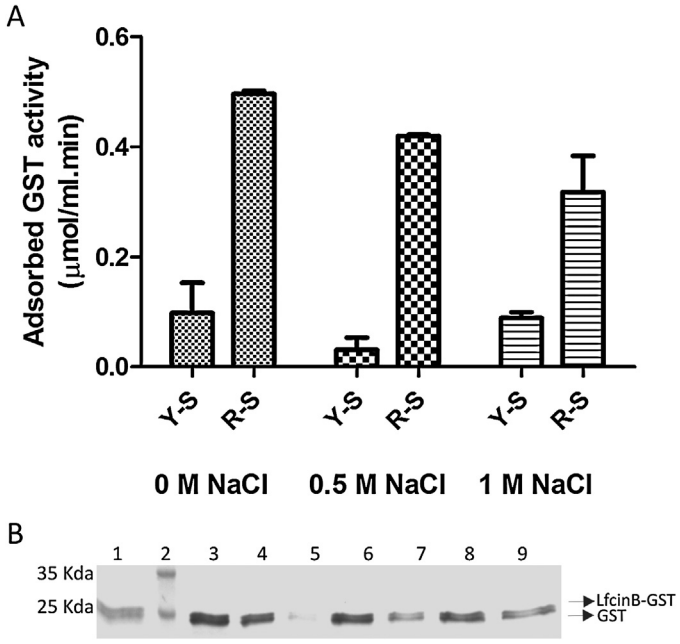

Fig. 7. Adsorption of GST to dye-Sepharose matrices. (A) Adsorption of GST to Yellow HE-4R-Sepharose matrix (Y-S) and to Red HE-3B-Sepharose matrix (R-S). Results are expressed by total adsorbed enzymatic GST units. (B) Analysis of adsorption of GST to Y-S and R-S using Western blot. Lane 1, expression of Lfcin B-GST in culture medium. Lane 2, protein ladder. Lane 3, expression of GST in culture medium. Lanes 4, 6 and 8, pass-through of Y-S without NaCl, with 0.5 M NaCl and with 1 M NaCl, respectively. Lanes 5, 7 and 9, pass-through of R-S without NaCl, with 0.5 M NaCl and with 1 M NaCl, respectively.

the dyes with the Lfcin B-GST fusion protein was specific for Lfcin B and not through GST, the same experiments were performed using a culture medium expressing GST (Fig. 5, lane 9). R-S showed a high affinity for GST even when the ionic strength of the culture medium was increased by the addition of 0.5 M NaCl or 1 M NaCl, whereas Y-S showed no affinity for GST in the same conditions (Fig. 7).

These results suggest that the interaction between Lfcin B-GST and Y-S is mediated by the Lfcin B fraction in the fusion protein. In the case of R-S, the interaction is mediated by GST and/or Lfcin B.

For further purification experiments, we selected Y-S because it was specific for Lfcin B-GST and avoided basal GST co-purification.

3.4. Optimization of the elution of Lfcin B-GST from the Yellow HE-4R-Sepharose matrix

Different eluents at different pHs were used for Lfcin B-GST desorption from Y-S (Table 2). The highest desorption values were obtained with 2 M NaCl with 25% ethylene glycol, at pH 9.0. A synergistic effect between NaCl and ethylene glycol was evident because the desorption values of NaCl or ethylene glycol alone were significantly lower (30% for NaCl and 2% for ethylene glycol at pH 9.0). This effect was also evident at pH 7.0 and pH 10.0. These results suggest that the affinity of Lfcin B-GST to Y-S is a result of both an electrostatic and a hydrophobic interaction. In addition, a pH value above the Lfcin B-GST isoelectric point (theoretical p*I* 8.6) impairs the electrostatic interactions and allows an increased desorption from the matrix.

Table 3 shows the performance of the purification process in different elution conditions with the Y-S matrix. The best condition was pH 9, where the purification factor was 18.0 and the yield of the fusion protein was 83.4 ± 0.3%. Fig. 8 shows a Western blot analysis of the purification process.

Table 2
Optimization of the elution of Lfcin B-GST from Yellow HE-4R Sepharose matrix.

Eluent	Elution (%)		
	pH 7.0	pH 9.0	pH 10.0
2 M NaCl	10.34 ± 0.75	30.66 ± 5.27	39.50 ± 4.32
2 M NaCl + 25% ethylene glycol	57.16 ± 2.13	83.42 ± 0.29	74.37 ± 3.86
0.5 M NaSCN	11.52 ± 0.50	16.50 ± 0.86	16.06 ± 1.10
0.5 M NaSCN + 25% ethylene glycol	22.23 ± 0.60	24.39 ± 2.99	30.07 ± 3.48
25% Ethylene glycol	1.88 ± 0.40	4.34 ± 0.60	8.34 ± 1.21

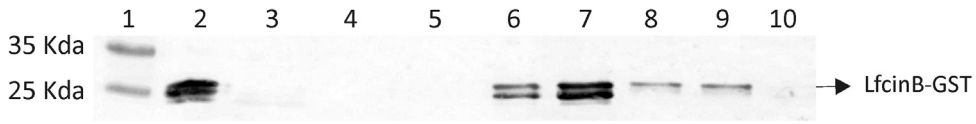

Fig. 8. Western blot analysis of the purification process of Lfcin B-GST using Yellow HE-4R-Sepharose matrix (Y-S). Lane 1, protein ladder. Lane 2, expression of Lfcin B-GST in culture medium. Lane 3, pass- through. Lanes 4 and 5, washes using 50 mM sodium phosphate buffer, pH 7.0, with 0.5 M NaCl. Lanes 6, 7, 8, 9 and 10, elution from Y-S using 50 mM Tris–HCl buffer at pH 9.0 with the addition of 2 M NaCl, 2 M NaCl with 25% ethylene glycol, 0.5 M NaSCN, 0.5 M NaSCN with 25% ethylene glycol or 25% ethylene glycol, respectively.

Table 3
Purification factor of the elution of Lfcin B-GST from Yellow HE-4R Sepharose matrix.

Eluent	Purification factor		
	pH 7.0	pH 9.0	pH 10.0
2 M NaCl + 25% ethylene glycol	9.6	18.0	8.3

Our findings evidence that Yellow HE-4R is a specific ligand for Lfcin B and was selected using SPR. To our knowledge, this is the first time that SPR is used to evaluate triazine dye–peptide interactions. SPR was a fast and high-precision tool to determine affinity parameters in the case of Lfcin B (it only has a secondary structure) but could also be used in other applications, including interaction between any other peptide and protein with a triazine dye. Moreover, SPR can replace the traditionally laborious method for dye screening. The approach of immobilizing the target peptide or protein to the chip of the surface plasmon resonance equipment takes advantage of the low quantities of this peptide or protein required and the drastic reduction of the time for dye ligand selection. The interaction of the immobilized Lfcin B with several triazinic dyes, which are abundant and cheap, could be assessed. On the contrary, the immobilization of the dye on the chip implies the use of several chips, one for each dye tested. This last strategy increases the costs of the selection process, is time consuming, laborious, and requires large quantities of the target peptide or protein [40].

The K_D values obtained for the interaction between the triazine dye Yellow HE-4R and Lfcin B by using SPR correlated well with the adsorption experiments using Y-S and the recombinant fusion protein Lfcin B-GST. The interaction between Lfcin B-GST and Y-S might be mediated by the Lfcin B fraction in the fusion protein. Also, the adsorption of Lfcin B-GST to Y-S, even with a high ionic strength, and the desorption using a combination of NaCl and ethylene glycol elucidated the electrostatic and hydrophobic interactions involved. Lfcin B (FKCRRWQWRMKKLGAPSITCVRRAF) presents an amphipathic secondary structure with hydrophobic and positively charged faces [13]. The arginine and lysine residues present in Lfcin B might be involved in the electrostatic interactions with Y-S, while the tryptophan residues might be involved in the hydrophobic interactions [13].

4. Conclusions

Lfcin B is an interesting peptide due to its various applications, but its recombinant expression is usually difficult and its downstream processing involves many steps. Herein, the Yellow HE-4R dye-selected by SPR – is described as a new and specific ligand for Lfcin B and its use for affinity chromatography becomes an attractive and low-cost strategy. In this work, recombinant Lfcin B expressed as a fusion protein with GST was recovered and purified directly from the baculovirus culture medium in only one step.

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