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Directed, Strong, and Reversible Immobilization of Proteins Tagged with a β-Trefoil Lectin Domain: A Simple Method to Immobilize Biomolecules on Plain Agarose Matrixes

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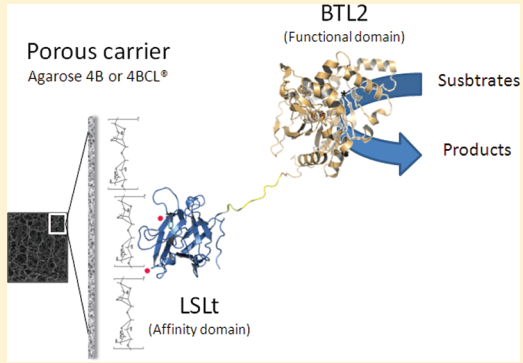
Supporting Information

ABSTRACT: A highly stable lipase from *Geobacillus thermocatenolatus* (BTL2) and the enhanced green fluorescent protein from *Aquorea victoria* (EGFP) were recombinantly produced N-terminally tagged to the lectin domain of the hemolytic pore-forming toxin LSLa from the mushroom *Laetiporus sulphureus*. Such a domain (LSL_{150}), recently described as a novel fusion tag, is based on a β-trefoil scaffold with two operative binding sites for galactose or galactose-containing derivatives. The fusion proteins herein analyzed have enabled us to characterize the binding mode of LSL_{150} to polymeric and solid substrates such as agarose beads. The lectin-fusion proteins are able to be quantitatively bound to both cross-linked and non-cross-linked agarose matrixes in a very rapid manner, resulting in a surprisingly dynamic protein distribution inside the porous beads that evolves from heterogeneous to homogeneous along the postimmobilization time. Such dynamic distribution can be related to the reversible nature of the LSL_{150}−agarose interaction. Furthermore, this latter interaction is temperature dependent since it is 4-fold stronger when the immobilization takes place at 25 °C than when it does at 4 °C. The strongest lectin−agarose interaction is also quite stable under a survey of different conditions such as high temperatures (up to 60 °C) or high organic solvent concentrations (up to 60% of acetonitrile). Notably, the use of cross-linked agarose would endow the system with more robustness due to its better mechanical properties compared to the noncross-linked one. The stability of the LSL_{150}−agarose interaction would prevent protein leaching during the operation process unless high pH media are used. In summary, we believe that the LSL_{150} lectin domain exhibits interesting structural features as an immobilization domain that makes it suitable to reversibly immobilize industrially relevant enzymes in very simple carriers as agarose.



INTRODUCTION

Enzyme immobilization has facilitated the process development of many biotransformations,[1,2] mainly because of the heterogeneous nature of the resulting biocatalysts. Such carrier-bound enzymes are easily and readily separated from the reaction mixture, enabling continuous operation.[3,4] There are many immobilization strategies that comprise reversible to irreversible attachments,[5−10] random to site-directed bindings,[11−13] and one-pot to several-pot methodologies.[8,14−16] Among all those immobilization technologies, specific immobilization of tagged proteins has been broadly used for many biotechnological applications.[17−19] Those tags may be either a small peptide of few aminoacids, such as the famous 6xHis-tag, or a larger and folded binding protein such as the glutathione *S*-transferase (26 kDa).[17] In general, all these systems reversibly and specifically immobilize the fusion protein readily expressed to be purified in one-pot.[20,21] In addition to the simplicity provided by these tags to the purification/immobilization protocols, it should be noted that many such affinity tags, overall those larger and folded ones, act as solubility enhancers increasing the production yield of soluble recombinant fusion protein.[22]

Lectin domains have been broadly applied in biosensor and microarray technologies, overall in the field of the glycobiotechnology.[23,24] Concanavalin A is one of the most used lectins to specifically immobilize glycoproteins; however, such a protein is normally attached to the solid surface rather than tagged to the target recombinant protein.[25,26] In this regard, we have recently described that the β-trefoil lectin domain of the pore-forming toxin LSLa from the mushroom *Laetiporus sulphureus* (LSL_{150})[27] exhibits properties typical of fusion tags which have permitted one to set up a novel protocol for the production and purification of recombinant proteins N-terminally tagged with such a domain.[28,29] This new protocol

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involves a simple, selective, and effective interaction between LSL_{150} and the chemical structure of polymerized agarose.[28,29] In this sense, the high resolution crystal structures of both unliganded LSL_{150} and LSL_{150} complexed with lactose permitted the detailed characterization of the sugar-binding mode of this lectin, revealing two lactose binding sites of the lectin as well as notable solvent rearrangements occurring upon lactose binding.[29] Therefore, it seems that the binding of LSL_{150} to agarose polymers must rely on those two functional sugar-binding sites.

Despite the large number of affinity tags currently described in the literature,[17,19] the vast majority of them have been applied in the recombinant protein production/purification process as chromatographic-based systems. Only a handful of them have been used as reversible immobilization platforms to make heterogeneous biocatalysts with industrial perspectives.[30−32] The immobilization via affinity tags enables the oriented immobilization of the fusion protein, with the linker between the N-terminal tag and the C-terminal companion acting as the spacer arm between the support surface and the catalytic domain, preserving the activity of the C-terminal enzyme within the immobilized composite.[30,33,34] Moreover, this methodology allows the one-pot purification/immobilization process because of the high selective interaction between the affinity tag and the carrier. One of the most representative examples for affinity immobilization is the 6x-His-tag system,[20,35] although few new methods have been developed in the past decade.[14,21,30] Nevertheless, many of these methodologies require functionalized carriers, for example, with metal chelates, alkyl-sulfates groups, glutathione, etc., increasing the cost and the complexity of the process.[18] On the contrary, in the system described in this work, the galactose-specific LSL_{150} domain is bound to nonactivated agarose matrixes which are more than 50 times less expensive than, for example, the manose−agarose matrixes needed for purifying recombinant MBP-tagged proteins.[28,36]

In this work, we make use of nonactivated commercial agarose (agarose 4B or agarose 4BCL) as carriers to specifically and reversibly immobilize two biotechnologically relevant proteins that have been produced as fusion proteins with LSL_{150} as the N-terminal tag: the cysteine-less mutant of the lipase from *Geobacillus thermocatenolatus*[37] (BTL2) and the enhanced green fluorescent protein from *Aquorea victoria* (EGFP). Optimal immobilization was achieved by contacting the carrier and the fusion proteins at 25 °C for at least 18 h. The interaction between LSL_{150} and the carrier was highly stable under drastic conditions such as 60% of organic solvent or 60 °C since the fusion protein remained bound to the carrier under those harsh conditions.

EXPERIMENTAL PROCEDURES

Materials. Agarose 4B and 4BCL were purchased from Agarose Bead Technologies (Madrid, Spain). Lactose was purchased from Sigma Chem. Co (St. Louis, USA). Restriction endonucleases and DNA polymerase were purchased from Fermentas International Co. and Biotools (Madrid, Spain), respectively. Other reagents were of analytical grade.

Cloning and Fusion Protein Production. The expression vector pKLSLt[29] was used as a template for the preparation of the plasmids pKLSLt-BTL2 and pKLSLt-EGFP coding for LSLt-BTL2 and LSLt-EGFP, respectively. As previously reported,[29] in pKLSLt the 3′-end of the LSL_{150} coding sequence is followed by an in-frame sequence coding for a flexible linker sequence (amino acids ASSS), the tobacco etch virus (TEV) endoprotease cleavage site (amino acids ENLYFQG), and a stop codon. That is, LSLt is LSL150 plus a four-residue linker and the TEV recognition sequence. Preparation of pKLSLt-BTL2 involved the amplification of the gene encoding for the lipase from *Geobacillus thermocatenolatus* (BTL2) by PCR with the PrimeSTAR HS DNA polymerase (Takara) using appropriate primers (forward, 5′-TGAAAACCTGTATTTCCAGGGCATGGCATCCC-CACGCGCCAA TG-3′; reverse, 5′-TCAGCTTCC-TTTCGGGCTTTGTTATTAAGGCCGCAAACTCGCCA AC-3′). The purified PCR product was then inserted into the pKLSLt vector by using the restriction-free and ligation-free cloning strategy previously described.[38] *Escherichia coli* DH5α cells were transformed, and recombinant plasmids and their sequences confirmed by DNA sequence analysis. Preparation of the pKLSLt-EGFP expression vector and production and purification of the fusion proteins were as indicated previously.[29] Briefly, the respective plasmids were used to transform *E. coli* BL21 (DE3) cells (Novagen, Germany). Transformed cells were grown at 37 °C in LB media containing 50 μg/mL kanamycin until the culture turbidity (OD_{600}) reached 0.6−0.8, when they were induced with 0.3 mM isopropyl-β-D-thiogalactoside (IPTG). After induction, the cultures were grown at 16 °C for 20 h before harvesting the cells by centrifugation at 4000*g* for 15 min. Cell pellets were suspended in 25 mL of 20 mM Tris-HCl, pH 8.0, and 100 mM NaCl and flash frozen at −80 °C until further use.

Purification of LSLt-BTL2 and LSLt-EGFP. The purification of LSLt-BTL2 and LSLt-EGFP was accomplished by affinity chromatography on Sepharose 4B at 4 °C using a BioLogic LP chromatography system (BioRad) and an Econo Gradient Pump (BioRad). Columns were prepared in-house using glass Econo-Column columns (2.5 × 10 cm; BioRad). Prior to sample loading, the resin was exhaustively washed with binding buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 0.04% (w/v) sodium azide). Cleared cell extracts were directly loaded onto the column at 2.5 mL/min, which was then washed with binding buffer overnight at 4 °C. Fusion proteins were then eluted with elution buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.04% sodium azide (w/v), and 0.2 M lactose) at 3 mL/min. Fractions containing the eluted fusion proteins were pooled and dialyzed overnight at 4 °C against binding buffer to remove bound lactose. Finally, polishing size-exclusion chromatography on 16/60 HiLoad Superdex 75 (GE Healthcare) was carried out to resolve the fusion proteins from potential soluble aggregates. Fractions were pooled and concentrated by ultrafiltration with YM-10 membranes (Amicon). Protein materials were stored at −80 °C. Protein purity was checked by SDS−PAGE. Regeneration of the Sepharose 4B column was done by washing with five volumes of elution buffer and then with five additional volumes of binding buffer.

Immobilization of LSLt-Tagged Proteins on Agarose-Type Carriers. Ten milliliters of 3 U/mL or 40 U/mL LSLt-BTL2 solution or 10 mL of 1 mg/mL LSLt-EGFP solution were incubated with 1 wet gram of either agarose 4B or 4BCL at different temperatures (4 or 25 °C) for different times. Supernatant and suspension samples were withdrawn at different times, and lipase activity of LSLt-BTL2 or protein concentration of LSLt-EGFP was determined according to the assay described by Fernández-Lorente et al.[39] and Bradford,[40] respectively.

Elution of Different LSLt-BTL2 Derivatives under Lactose Gradients. Different LSLt-BTL2 derivatives (0.1 g) were incubated for 30 min at pH 7 and 25 °C with 1 mL of lactose solution at different concentrations (0−400 mM). Activity of both supernatant and suspension was measured as described by Fernández-Lorente.[39]

Stability Studies of the LSLt Domain. LSLt-BTL2 derivatives immobilized on agarose 4BCL at 25 °C for 18 h were incubated at different postimmobilization conditions. Then, 0.1 g of such a derivative was incubated for 1 h with 1 mL of different buffer solutions at different pH values (50 mM sodium acetate, pH 5; 50 mM sodium phosphate, pH 7; 50 mM sodium carbonate, pH 10), at different temperatures (25, 45, and 60 °C), at different acetonitrile concentrations (20, 40, and 60%) and in the presence of 0.2% Triton X-100 at pH 7. Activity of both supernatant and suspension was measured as described by Fernández-Lorente.[39]

Confocal Laser Scanning Microscopy. Derivatives of LSLt-EGFP rapidly immobilized at 4 °C on either agarose 4B or 4BCL were resuspended in a ratio of 1/10 (w/v) (solid derivative/buffer). Postimmobilization fluorescence of such derivatives was monitored using an inverted Leica TCS SP5 laser confocal microscope with a 10× plan-apochromatic objective. Sequential scanning mode was used to avoid cross-talk between channels. All images shown correspond to a single confocal section. Images were processed with the LAS AF Leica application suite and Adobe Photoshop CS2 (Adobe Systems Inc.).

RESULTS AND DISCUSSION

Production and Characterization of LSLt-BTL2 and LSLt-EGFP. The cysteine-less mutant of the thermophilic lipase from *Geobacillus thermocatenolatus*[37] (BTL2) together with the enhanced green fluorescent protein from *Aquorea victoria* (EGFP) have been recombinantly produced N-terminally tagged with the fusion tag LSLt (see Experimental Procedures), resulting in the constructions LSLt-BTL2 and LSLt-EGFP, respectively. As we have recently described,[29] this novel fusion tag, based on the β-trefoil lectin domain of the pore-forming toxin LSLa from the mushroom *L. sulphureus*,[12] exhibits properties typical of both solubility enhancers and affinity tags, playing a passive role in the folding of its C-terminal partners. In this regard, LSLt-tagged EGFP showed the same spectroscopic behavior of untagged EGFP in solution, with a predominant fluorescence lifetime of 2.85 ns (see Table S1, Supporting Information);[41] moreover, the fluorescence decay of LSLt-tagged EGFP revealed the same lifetime components as those previously reported for free EGFP.[41] However, and as expected for the higher molecular weight of the fusion protein (45 kDa) relative to EGFP (27 kDa), the fluorescence anisotropy of LSLt-EGFP decayed more slowly than the free EGFP (Figure S1, Supporting Information). As a whole, these results show that LSLt does not affect the intrinsic spectroscopic behavior of EGFP, which agrees well with this tag playing no role in the folding of its C-terminal counterpart.

Likewise, the characterization of the hydrolytic activity of LSLt-BTL2 revealed that the genetic fusion of LSLt to the N-terminal end of the lipase did not affect its catalytic properties indicating both a proper folding of BTL2 and the lack of steric restraints imposed by the presence of the tag affecting the catalytic activity (Table 1). Moreover, LSLt-BTL2 was as stable as BTL2 (Figure S2, Supporting Information) when both soluble proteins were incubated at 45 °C. Moreover, the fusion of LSLt to reporter proteins has provided us for the first time with the opportunity to analyze the immobilization and elution behavior of such a lectin domain on agarose-type carriers even under drastic conditions. In the case of LSLt-BTL2, the highly stable enzymatic activity incorporated into the carrier could be spectrophotometrically monitored in an easy manner. In the same way, the microscopic distribution of the lectin domain within the pores of the carrier was also possible by monitoring LSLt-EGFP green fluorescence by a confocal scanning laser microscope (CLMS).

Table 1. Steady-State Kinetics Parameters of BTL2 Cysteine-Less Variant and the Same Variant Fused to a LSLt Domain

enzyme	V_{max} (U/mg)	K_m (mM)	K_{cat} (s^{-1})	K_{cat}/K_m ($s^{-1}\cdot M^{-1}\cdot 10^4$)
BTL2[a]	95.9 ± 0.2	1.46 ± 0.51	69.5 ± 0.2	4.7
LSLt-BTL2[b]	78 ± 3	1.2 ± 0.1	56.8 ± 2.2	4.8
Ag4BCL[c] (LSLt-BTL2)	75 ± 4	2.3 ± 0.1	54 ± 3	2.3

[a]This is the BTL2 variant where the two native cysteines were changed by two serines. [b]LSLt-BTL2. It is the cysteine-less BTL2 mutant N-terminally fused to LSLt. [c]Ag4BCL(LSLt-BTL2). It is the cysteine-less BTL2 mutant N-terminally fused to LSLt immobilized on plain agarose 4BCL.

Immobilization of Recombinant Proteins Fused to LSLt on Agarose Beads. LSLt-BTL2 and LSLt-EGFP were immobilized on two different types of agarose beads: agarose 4B and agarose 4BCL. The former is only polymerized agarose, whereas in the latter, the polymerized agarose has been cross-linked with epychloridrine in order to stabilize the agarose beads under harsh conditions. Noteworthy, both BTL2 and EGFP lacking the LSLt tag did not bind to either agarose 4B or 4BCL; therefore, immobilization specifically took place through interaction between the lectin domain and the agarose surface. Figure 1 shows the immobilization parameters (immobilization yield and expressed activity) of low loads of LSLt-BTL2 (30 $U\cdot g_{carrier}^{-1}$) on both agarose-based carriers. In both cases, the fusion protein was quantitatively bound to the matrixes apparently with similar immobilization rates (Figure 1A). Much higher loads of protein could be immobilized on plain-agarose beads; in fact, the maximum loading capacity of these matrixes was 43 mg of LSLt-BTL2 per g of wet matrix. Nevertheless, when immobilization conditions were forced (400 $U\cdot g_{carrier}^{-1}$), the immobilization rate was slightly higher for agarose 4B (Figure 1B). The different immobilization kinetics observed might rely on the particular chemical structures of the two agarose-type resins. Whereas agarose 4B keeps intact the polymeric structure of the agarose, the cross-linking treatment of agarose 4BCL alters the chemical structure of the β-1,3-D-galactose and α-1,3,4,6-anhydro-L-galactose monomers found in the agarose polymer, and consequently, the lectin-agarose 4BCL interaction might be affected.

Surprisingly, the expressed activity of the immobilized lipase decreased as a result of the immobilization process. The final expressed activities were 60 and 50% of the LSLt-BTL2 activity in solution, when the protein was immobilized on agarose 4B and 4BCL, respectively (Figure 1A). Typically, reduced values of enzymatic activities upon enzyme immobilization can be explained in terms of protein inactivation promoted by the immobilization protocol or to the existence of a rate-limiting diffusion of the substrate into the porous carrier. We believe that, in our case, the first possibility can be discarded because, *a*

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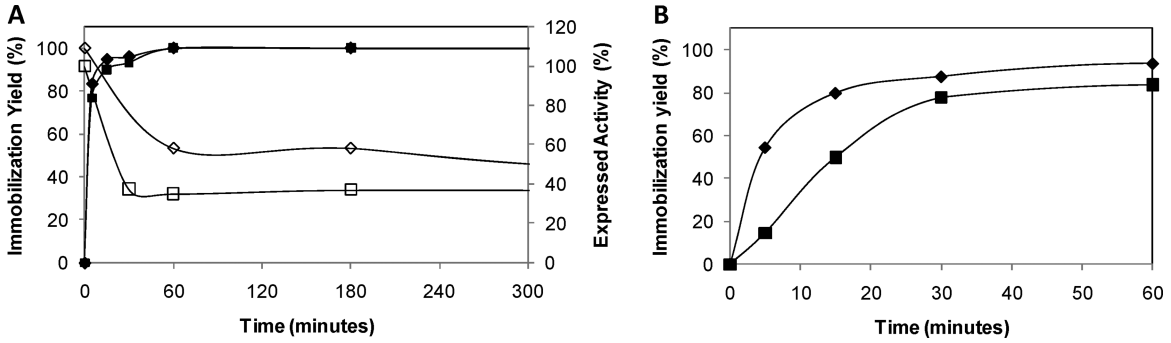

Figure 1. Immobilization of LSLt-BTL2 on different agarose type matrixes. LSLt-BTL2 was immobilized on agarose 4B (rhombus) and on agarose 4BCL (squares). Immobilization yield (closed symbols) and expressed activity (open symbols) were calculated for each immobilization experiment. (A) The immobilization was carried out by incubating 30 U of LSLt-BTL2 activity per gram of support in an immobilization ratio of 0,25 m^2 of support per mL of immobilization mixture. (B) Immobilization was carried out incubating 400 U of LSLt-BTL2 activity per gram of support in an immobilization ratio of 2.5 m^2 of support per mL of immobilization mixture. In both cases, immobilization occurred at pH 7 and 25 °C. Immobilization patterns were quite similar between both types of agarose matrixes, and only when immobilization conditions were forced to immobilize high amounts of enzyme, a slight faster immobilization could be observed with agarose 4B. Data corresponded to the mean value of three independent experiments where error was never higher than 5%.

priori, the LSLt-BTL2 orientation on the support surface (the N-terminal end of the lipase, and therefore the LSLt tag, is located on the opposite site of the active center of the enzyme) is favorable to avoid a direct interaction between the lipase active center and the support surface that might affect the binding pocket conformation (Figure 2). Hence, it seems

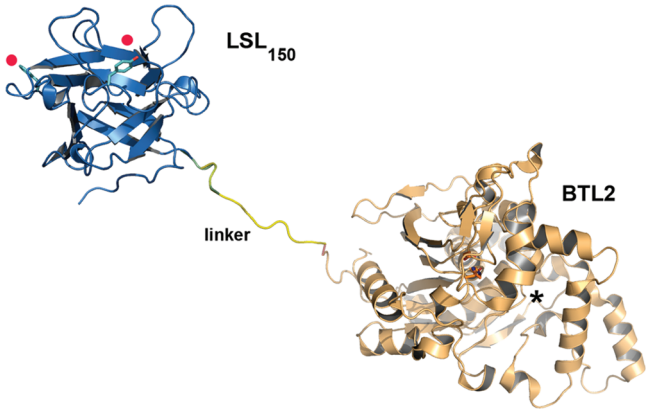

Figure 2. Putative atomic model of LSLt-BTL2. This tentative model has been built considering the crystal structures of both LSL_{150} (PDB code: 2Y9F) and BTL2 (PDB code: 2W22), with an extended linker containing 11 amino acid residues to provide a flexible region between the N-terminal lectin module that directly interacts with the matrixes and the accompanying C-terminal lipase. The two red circles indicate the β and γ sugar-binding sites (Tyr-91 from the β site and Phe-139 from the γ site, shown as sticks), and the asterisk indicates the entrance to the active site (the residues that make up the catalytic triad are also shown as sticks).

plausible to think that reduction of the expressed activity was due to rate-limiting substrate diffusion into the carrier since the activity measurements were carried out under nonsaturating substrate (500 μM pNPB) conditions because it was quite insoluble in aqueous media. In fact, the eluted activity per gram of support of the LSLt-BTL2 derivatives incubated for 24 h with 400 mM of lactose was roughly double the expressed activity of the immobilized protein per gram and almost the same as the immobilized activity per gram (Table S2, Supporting Information). Moreover, the apparent K_m value of the immobilized protein was increased by a factor of 2 (Table 1). These facts support the idea that LSLt-BTL2 in its immobilized form was subjected to higher mass transfer restrictions than its soluble one. One of the main reasons that would explain the diffusional limitations observed for well-oriented and nondeactivated enzymes immobilized on porous carriers would be their distribution throughout the porous surface.[42,43] It has been reported that heterogeneous enzyme distribution leads to a much better catalytic effectiveness under conditions of mass transfer limitations than those enzymes homogeneously distributed across the porous surface.[43] Hence, in the heterogeneous systems, the higher effectiveness would rely on the easier diffusivity of the reactants to the enzyme catalytic centers. In our case, the protein distribution is essentially driven by the interaction between the lectin module of the fusion protein and the agarose surface. In order to study the protein distribution within the carrier, LSLt-EGFP was immobilized on both agarose 4B and 4BCL, and the resulting derivatives were analyzed by CLMS. Surprisingly, a homogeneous distribution of LSLt-EGFP was found in both agarose 4B and 4BCL, which *a priori* does not agree with the fact that proteins rapidly immobilized show heterogeneous distributions, being mainly located at the bead outline.[44] To shed light on this apparent discrepancy, we dynamically studied the post-immobilization localization of LSLt-EGFP. First, LSLt-EGFP was rapidly immobilized on both agarose 4B and 4BCL at 4 °C, and after 1 min, the immobilization suspension was filtered and washed to remove unbound protein. Finally, beads containing immobilized LSLt-EGFP were diluted in aqueous media, and their fluorescence was recorded at various time intervals. Interestingly, it could be observed how proteins migrated inside the porous surface during postimmobilization incubation (Figure 3A). At short postimmobilization times, LSLt-EGFP was mainly heterogeneously distributed colonizing only the outer surface of the beads, while at longer postimmobilization times, LSLt-EGFP colonized the whole porous surface revealing a homogeneous protein distribution, which was stable in the time scale herein consider (videos S1 and S2, Supporting Information). This dynamic behavior of the LSLt-EGFP distribution presumably reveals an underlying LSLt-agarose binding mode based on the rapid and reversible association/dissociation process inside the pores. Although this system is

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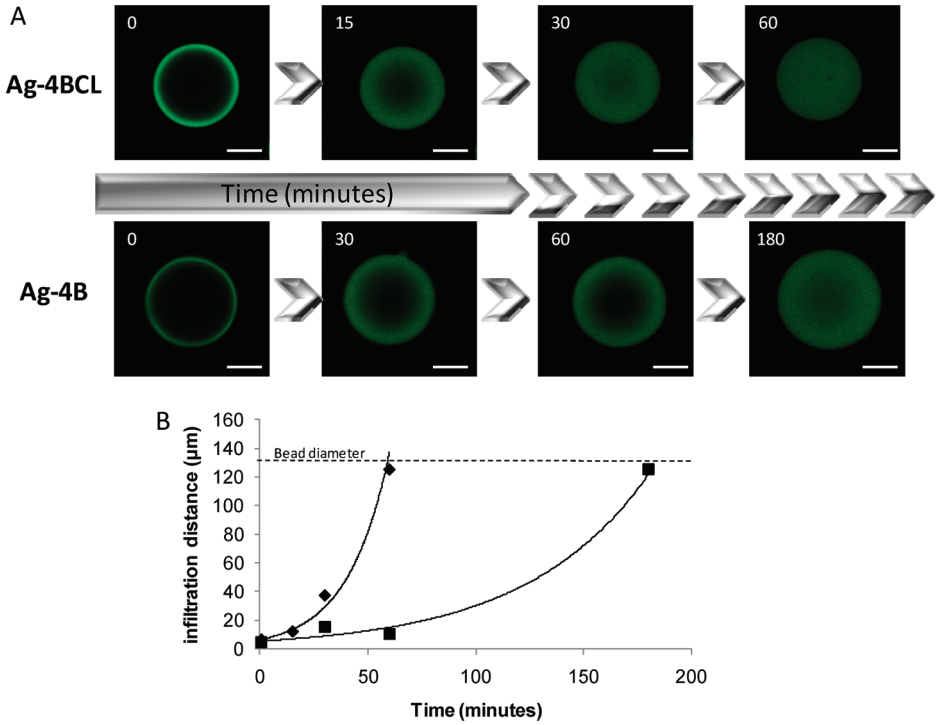

Figure 3. Time-dependence of the spatial distribution of LSLt-EGFP inside different agarose type matrixes. The immobilizations were carried out by incubation of 1 mg of LSLt-EGFP per gram of each support. When LSLt-EGFP was quantitative bound, the derivatives were vacuum filtered and resuspended in 25 mM sodium phosphate buffer, pH 7, and subsequently analyzed by CLMS. (A) CLMS images recorded at different time intervals. (B) The infiltration distance of LSLt-EGFP was calculated at different times by measuring the size of the radius occupied by 90% of the total fluorescence given by such a protein on agarose 4B (rhombus) and on agarose 4BCL (squares). The experimental data were fitted to the mathematical equation $D_i = A \cdot e^{\sqrt{Vd \cdot t}}$ in order to determine the distribution velocity (V_d). It can be observed how in both agaroses the LSLt-EGFP was infiltrated deeper and deeper through the porous surface along time. The full homogeneous distribution took longer when the immobilization carrier was agarose 4B. The size of the agarose beads represented in this figure is roughly 125 μm, and the white scale bar is 50 μm.

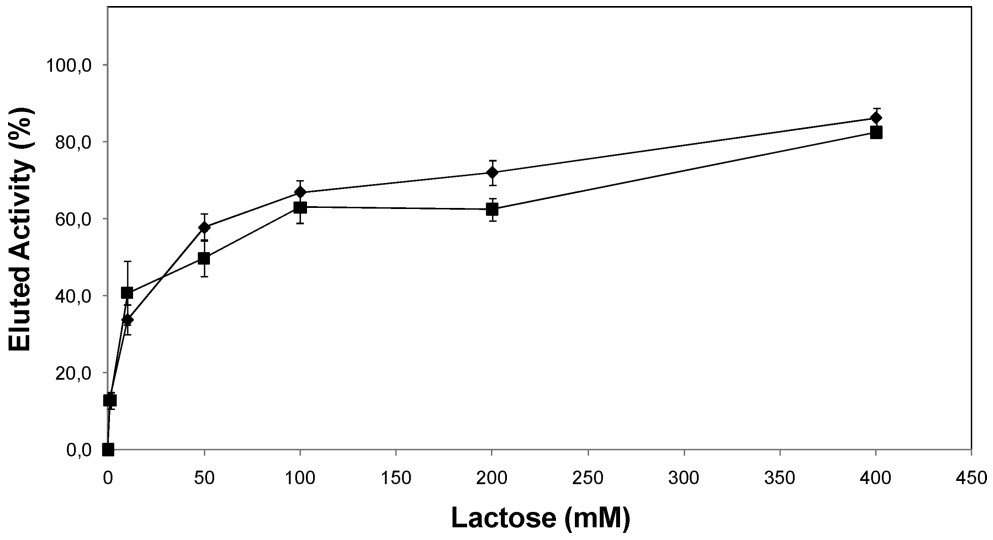

Figure 4. Binding strength of the interaction between the LSLt domain and different types of agarose matrixes. LSLt-BTL2 immobilized on agarose matrixes at 4 °C for 15 min (100% of immobilization) was eluted with different concentrations of lactose (0–400 mM). This experiment allows us to evaluate the apparent affinity of the LSLt domain to the galactose units forming the agarose structure. The two studied matrixes were agarose 4B (rhombus) and agarose 4BCL (squares). The data shown are the means of three independent experiments with SD (±) represented as error bars.

shifted to the associated state, which allows quantitative immobilization, the reversible nature of the interaction would enable the observed protein migration from a heterogeneous distribution to a homogeneous one. This migration from crowded regions to less busy ones would be possible because of the higher availability of galactose-type affinity groups in the noncolonized areas. In this context, the proteins dissociated within the agarose particles would tend to interact with noncolonized areas inside the porous structure where the density of the galactose-type groups is higher, and therefore, the association would be favored. Noteworthy, the rate of LSLt-EGFP distribution throughout the porous surface was almost 3-

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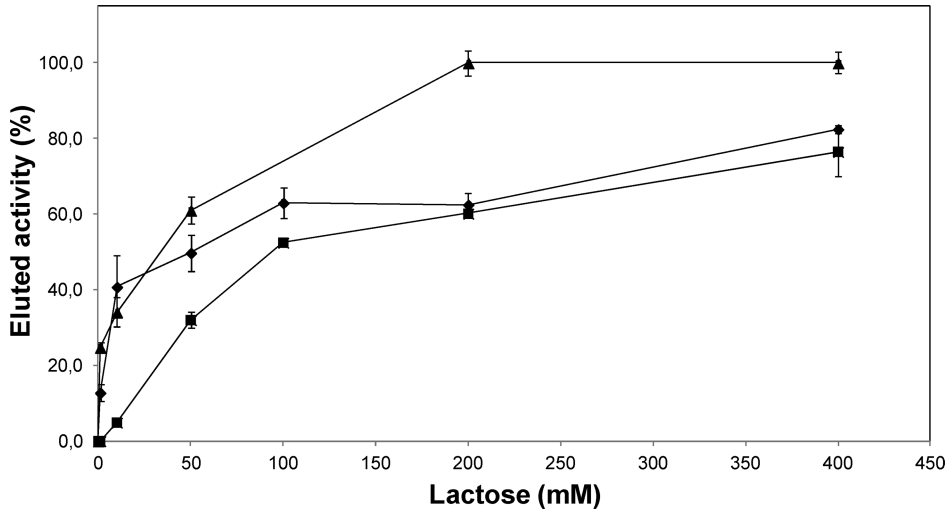

Figure 5. Effect of temperature and time of immobilization on the binding strength of the LSLt domain to agarose 4BCL. Different immobilized derivatives of LSLt-BTL2 on agarose 4BCL were eluted under different concentrations of lactose (0–400 mM). This experiment allows us to evaluate how strong the LSLt domain is bound to the galactose units forming the agarose 4BCL structure. The immobilizations were prepared at two different temperatures, 25 °C (squares) and 4 °C (triangles), for 18 h, observing a higher binding strength when the immobilization was carried out at higher temperature. LSLt-BTL2 was also immobilized at 25 °C for 15 min (rhombus) observing that the first elution fraction was weakly bound for short immobilization times than for longer ones. Data correspond to the mean value of three independent experiments with SD (±) represented as error bars.

fold faster when the fusion protein was immobilized on agarose 4BCL than when it was on agarose 4B (Figure 3B and Table S3, Supporting Information). In this regard, the different chemical structure of both agarose matrixes might be affecting to the association/dissociation equilibrium of the lectin-agarose binding. Hence, at least at short postimmobilization times, the lectin domain seemed to be more easily dissociated from agarose 4BCL than from 4B since LSLt-EGFP distribution evolved faster in the former agarose (video S1, Supporting Information) than in the latter (video S2, Supporting Information) (Figure S3 and Table S3, Supporting Information).

Therefore, it can be concluded that the immobilization of LSLt-tagged proteins on agarose-based matrixes is highly rapid, yielding homogeneous protein distributions. As far as we know, this is the first time where homogeneous protein distributions throughout porous surfaces has been achieved by immobilizing low loads of one protein. It was possible because of the postimmobilization migration experienced by the immobilized proteins (Figure S3, Supporting Information). This particular feature has many potential applications such as new lab-on-a-chip devices to detect important analytes in medical or environmental chemistry[45] or in the ordered assembly of biomolecular platforms inside porous materials in combination with other immobilization chemistries that promote heterogeneous protein distribution.[46,47]

Reversibility of the LSLt–Agarose Interaction: Effect of Different Variables on the Strength of the Lectin–Agarose Interaction. As shown above, the interaction between LSLt and different agarose matrixes occurs through a reversible interaction; in fact, fusion proteins can be eluted by incubation at high lactose concentrations during the purification protocol.[28,29] Now, we have further analyzed this dissociation process by studying its dependence on lactose concentration, which in turn allows us to evaluate the apparent affinity of LSLt toward the agarose beads system. Figure 4 shows that LSLt-BTL2 was similarly eluted from both agarose 4B and 4BCL, suggesting that LSLt binds to both matrixes with the same strength, at least at the macroscopic level. Since cross-linked carriers provide better mechanical properties, allowing biocatalysts to work under more drastic operational conditions, we believe that this result may open up applications of LSLt in applied biocatalysis as a generic, immobilizing domain of proteins on agarose-based carriers.

In addition, we have also analyzed the effect of temperature and time of immobilization on the binding strength of LSLt. With this aim, LSLt-BTL2 was immobilized on agarose 4BCL for different times (15 min and 18 h) at different temperatures (4 and 25 °C), and the immobilized derivatives were then incubated with different lactose concentrations. Temperature was revealed as the most important parameter affecting the strength of the lectin–agarose bead interaction (Figure 5). For instance, the 60% of the LSLt-BTL2 immobilized at 4 °C for 18 h was eluted with 50 mM lactose, whereas at least 200 mM lactose was needed to elute the same activity percentage of LSLt-BTL2 immobilized at 25 °C for the same time (Figure 5); it would mean a 4-fold harder elution by carrying out the immobilization at 25 °C. The same effect was observed for derivatives immobilized only for 15 min but at different temperatures. Although the incubation time did not affect as much as the temperature to the binding strength, it is interesting to highlight how for the derivatives immobilized for 15 min at 25 °C, the earlier eluted fractions needed lower lactose concentration than the same fraction from derivatives immobilized for longer times at the same temperature (Figure 5).

The temperature-based versatility of the binding strength of the lectin–agarose interaction would make this system suitable only for either protein purification or one-pot protein purification/immobilization. In this way, for purification purposes strong binding would not be desired because the protein must be eluted later, whereas for applied purification/immobilization protocols, strong binding would be preferred in order to avoid the protein leaching during the operation

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process. Hence, by switching the immobilization conditions from low temperatures and short immobilization times to medium temperatures and longer incubation times, the purification methodology would become useful for immobilizing proteins.

Stability of LSLt Domain under Different Conditions. The immobilization of LSLt-fusion proteins on agarose matrixes relies on its sugar-binding properties, which in turn depends on its β-trefoil structure, in particular, on the native state of its two operative sugar-binding sites.[48] Hence, full or partial unfolding of the LSLt domain would promote the release of the fusion protein from the solid composite. In applied biocatalysis, the reaction conditions may be quite drastic and denaturing for protein structures. In order to evaluate the stability of the LSLt domain tertiary structure, the optimal derivative of LSLt-BTL2 immobilized on agarose 4BCL was incubated under different conditions of pH, temperature, and the presence of organic solvents. It is important to note that since soluble LSLt-BTL2 retained 100% of its initial activity under all studied conditions due to its intrinsic high stability, the obtained results depended exclusively on the stability of the LSLt domain. Table 2 shows that the LSLt domain was highly stable against temperature and tolerated relatively high concentrations of acetonitrile (more than 90% of immobilized enzyme remained on the carrier after incubation at up to 60 °C or up to 60% of acetonitrile). Nevertheless, LSLt−agarose bead binding was seriously affected by alkaline conditions because the fusion protein was totally released from the carrier. Nonionic surfactans such as Triton X-100 also disrupted the interaction between LSLt and the agarose beads. Therefore, alkaline conditions and detergents seemed to promote the β-trefoil structural distortions that lead to the dissociation of the lectin domain from the agarose matrix. However, in spite of the low stability of the LSLt domain in these latter conditions, this methodology is quite robust for a large variety of reaction conditions. Immobilization based on the use of LSLt may be used in fine chemistry reactions where substrates often present solubility issues, and organic solvents are required in the reaction media. Furthermore, enzyme derivatives immobilized via lectin might be also used in reaction environments where medium-high temperatures (40−60 °C) are needed, as, for example, in food chemistry for lactose-free processes where elevated temperatures are used to avoid microbial contaminations. Therefore, the robustness of LSLt−agarose bead interaction, the simplicity of the one-pot purification/immobilization protocol, and the relative low cost of the nonactivated agarose carriers present this methodology as a new opportunity to prepare cost-effective immobilized biocatalysts with attractive industrial perspectives.

Table 2. Elution of LSLt-BTL from Agarose 4BCL under Different Conditions

elution conditions[a]	eluted BTL2 activity (%)[b]
buffer sodium phosphate, pH 7, 25 °C	**0**
buffer sodium phosphate, pH 7, 45 °C	**7**
buffer sodium phosphate, pH 7, 60 °C	**3**
buffer sodium acetate, pH 5, 25 °C	**20**
buffer sodium carbonate, pH 10, 25 °C	**100**
buffer sodium phosphate, pH 7, 25 °C, Triton X-100 0,2%	**60**
acetonitrile 20%, pH 7, 25 °C	**10**
acetonitrile 40%, pH 7, 25 °C	**0**
acetonitrile 60%, pH 7, 25 °C	**5**

[a]Under all elution conditions, the soluble LSLt-BTL2 kept 100% of its initial activity. [b]Eluted BTL2 activity is defined as the lipase activity measured with *p*NPB (see methods) in the supernatant regarding the immobilized activity before the immobilized enzyme was incubated at different conditions. The eluted activities are the mean of three independent experiments, and the error was never higher to 10%.

CONCLUSIONS

The LSLt domain was genetically fused to the N-terminal end of two different proteins: cysteine-less mutant of BTL2, a thermostable lipase, and EGFP, an excellent reporter protein. The resulting fusion proteins were immobilized onto different agarose-type carriers via affinity interaction between such lectin domains and the galactose units present in the structure of the agarose beads. The fusion of catalytic and reporter proteins to the LSLt domain allowed the study of the immobilization kinetics of this system revealing a novel mechanism involving a rapid immobilization step on both noncross-linked and cross-linked agarose matrixes followed by a dynamic distribution of the proteins throughout the porous surface. Slight differences were observed in the binding of LSLt to either agarose 4B or 4BCL, indicating that binding was slightly more favorable for the former one. Furthermore, the interaction between the lectin and agarose was temperature-dependent since the binding was 4-fold stronger when the immobilization took place at 25 °C than when it did at 4 °C. The strongest interaction, lectin−agarose, was also quite stable under a survey of different conditions such as high temperatures (up to 60 °C) or high organic solvent concentrations (up to 60% of acetonitrile). Hence, this lectin domain seems to be suitable to reversibly immobilize enzymes industrially relevant in very simple carriers as agarose. Although immobilization was a little more efficient on noncross-liked agaroses, the use of cross-linked ones is more interesting because of their better mechanical properties. This immobilization also enables the one-pot purification and immobilization thanks to the highly specific and strong interaction between the lectin domain and the agarose surface.

ASSOCIATED CONTENT

Supporting Information

Experimental procedures II; spectroscopic behavior of both EGFP and LSLt-EGFP; effect of the LSLt tag on the final stability of the soluble fusion protein; determination of diffusion restriction observed during the immobilization process of LSLt-BTL2 on agarose matrixes; and dynamic distribution of LStL-GFP throughout agarose porous surface. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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