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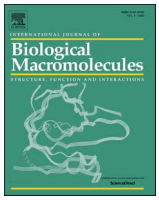

Tailoring the specificity of ficin versus large hemoglobin and small casein by co-immobilizing inert proteins on the immobilized enzyme layer and further modification with aldehyde dextran

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ABSTRACT

Ficin has been immobilized at full loading on glyoxyl agarose beads. Then, ficin was blocked with 2,2′-dipyridyldisulfide. To be effective, the modification must be performed in the presence of 0.5 M urea, as the enzyme was not inhibited under standard conditions, very likely because the catalytic Cys was not fully exposed to the medium. Activity could be fully recovered by incubation with 1 M mercaptoethanol. This biocatalyst could hydrolyze hemoglobin and casein. The objective of this paper was to increase the enzyme specificity versus small proteins by generating steric hindrances to the access of large proteins. The step by step blocking via ionic exchange of the biocatalyst with aminated bovine serum albumin (BSA), aldehyde dextran and a second layer of aminated BSA produced a biocatalyst that maintained its activity versus small synthetic substrates, increased the biocatalyst stability, while reduced its activity to over 50 % versus casein. Interestingly, this treatment almost fully annulled the activity versus hemoglobin, more effectively at 37 °C than at 55 °C. The biocatalyst could be reused 5 times without changes in activity. The changes could be caused by steric hindrances, but it cannot be discarded some changes in enzyme sequence specificity caused by the modifications.

1. Introduction

Proteases are enzymes with a great interest for different applications [1–6]. They can be utilized to produce bioactive peptides [7–10], in fabrics refinement [11–13], milk clogging [14–17], tenderizing meats [18–20], or as antimicrobial agents [21] (e.g., versus biofilm formation [22,23]), to cite a few examples. That way, proteases have multiple possible utilizations, from being detergent components [24,25] to clinical applications [1].

The immobilization of proteases may simplify their utilization in many instances [26,27]. Nowadays, the recover and reuse of enzymes, whose price is decreasing, may not justify the enzyme immobilization. Only if the presence of the enzyme in the reaction medium makes the product downstream complex, or, even more interestingly, if the enzyme properties are significantly improved by immobilization, it may be fully justified [28–33]. One immobilized protease molecule cannot attack to other immobilized enzyme molecule, that way autolysis is no longer possible [1]. Immobilized proteases may be applied under free enzyme precipitation conditions [34]. If immobilization produces a more rigid protease structure, e.g., via multipoint covalent attachment, the biocatalyst can be applied under more drastic conditions (such as higher temperatures, presence of caotropic agents necessary to re-dissolve protein aggregates, etc.) increasing the possibilities of achieving a suitable biocatalyst for a particular process [34]. Enzyme immobilization may also overcome inhibition matters, in fact, the use of a protease was the first report on eliminating a substrate inhibition matter via immobilization to be published [30,31,35]. Enzyme purification may be coupled to immobilization using adequately designed immobilization protocols [36]. Enzyme specificity and selectivity may be also changed, due to the conformational changes usually associated to the enzyme-support interactions and the fact of locating the enzyme in a confined place [37]. Molecular simulation is currently helping to predict the

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effects of the immobilization on the final enzyme properties [38]. There are multiple strategies to immobilize enzymes [39–43] in this paper will focus on enzyme immobilization on pre-existing supports [31]. That way, enzyme (and protease) immobilization may be considered a discipline in a continuous evolution [44].

In this new research effort, we have focused on a new challenge. We intend to change the specificity of proteases regarding the size of the protein substrate, preparing a biocatalyst that mainly hydrolyzes small proteins while not modifying large ones. As far as we know, this is the first published attempt to reach this objective. The idea is to prepare an immobilized protease biocatalyst that can be able to hydrolyze proteins of a size similar or smaller to the protease, but that cannot modify proteins significantly larger than the employed protease. Theoretically, immobilized protease with an orientation that produces a protease molecule with the active center near the surface of the support but not fully blocked, may permit the protease to only recognize significantly small proteins, but not proteins as large as the immobilized protease [45]. In any case, this strict protease orientation on the support surface requires the use of genetic manipulations of the enzyme, and it has not been reported in the literature.

In this paper, we have assayed a fully different and novel strategy. Using properly immobilized proteases (the active center is not oriented to the matrix surface) with high capacity to hydrolyze large and small proteins, we intend to design steric hindrances for the access of proteins to the active center of the proteases in a way that only small proteins can reach the active center while large ones will be unable, and therefore remain unmodified. To generate these steric hindrances, after immobilizing the protease at maximum support loading, we will immobilize a layer of a non-catalytic protein, with the aim of impeding the access of the substrate protein to the protease active center by increasing the tortuosity of the pathway of the substrate, and expecting that this can more significantly affect the access of large than of small proteins to the active center of the enzyme [46–51]. The number of inert protein layers may be increased if necessary. That way, it will be possible to use the best protocol to immobilize the protease in terms of stability and activity even if the target is to discriminate between large and small proteins. This strategy should be valid for any immobilized enzyme intended to modify moderately small substrates in the presence of large ones that must not be modified. In a chromatographic context, a similar strategy was developed by Prof. Guisańs group many years ago to design an ionic exchange matrix unable to adsorb large proteins but able to absorb small ones [52].

As model system where try the proof of concept, ficin extract (composed by several proteases) immobilized on glyoxyl agarose has been chosen. Ficin extraxct immobilization on glyoxyl agarose generates a biocatalyst with an improved enzyme stability [53–56] (with some limits due to oxidation of the catalytic Cys [57]), and it can be utilized for milk clogging with very high clog yield [58]. This ficin extract biocatalysts can hydrolyze casein to free amino acids and dipeptides after 24 h of reactions, showing the wide specificity of the components of the extract, This thiol protease can be reversibly inhibited using a reactive disulfide, and the activity may be recovered when desired [57,59]. This is important because using an active immobilized protease; it may be difficult to prevent that it can hydrolyze the inert protein that we intend to coimmobilize over the protease layer, making controlling the protein layer over the protease layer difficult. This may be a new application of the possibility of sulfidryl proteases switch off and switch on by using proper inhibitors [59]. As a non-catalytic protein, bovine serum albumin has been selected; in fact, the protein has been aminated using the carbodiimide route [60–63] to reach the rapid adsorption via ion exchange on the immobilized ficin (that has an elevated isoelectric point) [64], and also to have a high reactivity with the selected inert protein layers connector. As an inert protein layer connector, dextran aldehyde has been selected [65]. A size of 40,000 D dextran has been utilized in this approach. Aldehyde dextran has been used (after modification of the target support/biocatalyst and further reduction after fulfil its function) as inert support coating agent on many instances [52,66,67]. This way, the adsorption of the substrate proteins on the biocatalyst will be minimized. It is expected that the random coil nature of this compound may help to increase the tortuosity of the pathway to the active center of the immobilized ficin, without fully blocking the access to small proteins. As model substrate proteins, we have chosen casein as small protein (around 23 KD, similar to the size of ficin (around 21 KD)) and hemoglobin, (a tetrameric protein of 64 KD) as a model of large protein.

2. Materials and methods

2.1. Materials

2,2′-dipyridyldisulfide (2PDS) and hemoglobin were bought from Thermo-Fisher (Kandel, Germany)Cysteine, 2-mercaptoethanol, ethylenediaminetetraacetic acid (EDTA), benzoyl-arginine-p-nitroanilide (BAPNA), casein, glycidol, sodium periodate and sodium borohydride were purchased from Sigma-Aldrich (St. Louis, MO, USA). Agarose beads 4 BCL support was bought from Agarose Bead Technologies (ABT), (Alcobendas, Spain). Glyoxyl agarose beads were prepared as previously reported [68,69]. All other reagents were of analytical grade.

2.2. Methods

2.2.1. Ficin preparation

Ficus carica L latex was obtained and placed in a flask in Adekar, Bejaia in the Northam of Algeria. This sample was stored at 4 °C. The sample was centrifuged (10,000 *g* at 4 °C for 15 min) to discard solid components and stowed at −20 °C. This supernatant (with a protein concentration of 78 mg /mL) was employed as ficin extract in all experiments described in this paper. Bradford's method [70], utilizing BSA as reference, was employed to determine the protein concentration.

2.2.2. Amination of BSA with EDC and EDA

1 g of BSA was added to 50 mL of a solution composed of 1 M EDA at pH 4.75. Next, solid EDC was added until reaching a concentration of 10 mM and the reaction was maintained for 90 min of gentle stirring at 25 °C. Under these conditions, full modification of exposed carboxylic groups is obtained [60].

Next, the aminated BSA was dialyzed at 4 °C for 48 h employing a membrane with a molecular weight cutoff of 10 kD. This process was performed in 2.5 L of 5 mM phosphate buffer, with changes in the buffer after 2 h, 4 h, 8 h, 18 h, 24 h, 36 h and 48 h.

2.2.3. Preparation of aldehyde dextran

Aldehyde dextran (40,000 Da) [65] was produced by full oxidation with sodium periodate as previously reported by Betancor et al., 2004 [71]. 6.66 g of dextran were dissolved in 200 mL of distilled water. Then, 16 g of sodium periodate was added at 25 °C under continuous stirring, and the reaction was leave to proceed for 3 h. Next, the solution was dialyzed against 50 volumes of distilled water, the dialysis water was changed after 2 h, 4 h, 8 h, 18 h and 24 h. The obtained aldehyde dextran was storage at 6 °C before use.

2.2.4. Enzymatic assays

Ficin biocatalysts activity was quantified by utilizing BAPNA, casein or hemoglobin as substrates.

BAPNA assay was carried out following the method reported by Siar et al. [54]. To 1.0 mL of dimethyl sulfoxide, 43.5 mg of BAPNA were added. Next, 99 mL in 0.1 M sodium phosphate pH 7.0, containing 5 mM cysteine and 5 mM EDTA was added. The activity assay was performed by determining the increase in absorbance at 405 nm promoted by the release of *p*-nitroaniline (the ε was 8800 under these conditions) using a Jasco V 730 (Madrid, Spain) spectrophotometer with control of temperature and magnetic stirring. One activity unit is given in micromoles of produced *p*-nitroaniline per minute.

Casein and hemoglobin hydrolyses were carried out according to the protocol reported by Kunitz [72] with minor modifications. Casein or hemoglobin was dissolved at 1 % (*w/v*) in 100 mM sodium phosphate containing 5 mM EDTA and 5 mM cysteine, and the pH was adjusted to 7.0. This solution was heated to the desired temperature (the standard one was 55 °C). To initialize the reaction, 200 μL of biocatalysts suspension or solution was added to 1 mL of substrate solution. This reaction mixture was incubated at the desired temperature at different times (30 min is the standard assay). To finalize the reaction, 2 mL of 10 % trichloroacetic acid (TCA) was added, producing the precipitation of the non-hydrolyzed protein, but the small peptides produced by the hydrolysis of proteins remain in solution. This suspension was centrifuged at 10,000 rpm and the absorbance of the supernatant was quantified at 280 nm. As a reference, substrate was added enzyme inactivation by incubation in TCA. As unit of protease activity, we utilize augmentation in the absorbance by 0.001 min^{-1}. In some instances, to have more complete reaction courses, the reaction time was prolonged.

2.2.5. Immobilization of ficin extract on glyoxyl agarose beads

Ficin was immobilized on glyoxyl-agarose beads by adding to 200 mL of a solution 50 mM sodium carbonate, 8 mg/mL ficin extract solution. The pH was adjusted to pH 10.05, and 20 g of glyoxyl agarose (this enzyme loading slightly exceeded the maximum loading capacity of the support) were added. This suspension was submitted to continuous stirring at room temperature [57] for 24 h [54]. Part of the suspension was reduced by adding solid $NaBH_4$ (to a concentration of 1 mg/mL), for 30 min at room temperature maintaining the gentle stirring. The produced ficin biocatalysts were washed with an excess of distilled water. Immobilization yield (Eq. (1)) refers to the percentage of offered enzyme that become immobilized on the support, while expressed activity refers to the ratio between experiential activities and expected immobilized activities from the immobilization yield [33].

$$\text{Immobilization yield} = \text{activity of supernatant/activity of reference} \quad (1)$$

$$\text{Expressed activity} = \text{Obserbed activity/expected activity from immobilization yield} \quad (2)$$

2.2.6. Ficin modification with 2PDS

A solution of 5 mM 2 PDS in 5 mM sodium phosphate containing different concentrations of urea (0.5, 0.75, 1, 2 and 3 M) and 10 % dimethyl sulfoxide (DMSO) at pH 7 was prepared. The aim of this experiment was to block the catalytic Cys residue [57,59]. Then, glyoxyl-ficin biocatalyst was added and the suspension was submitted to constant stirring at room temperature for 4 h. Then, the inhibited biocatalysts recovered by filtration and washed with abundant distilled water.

2.2.7. Coimmobilization of BSA over the immobilized and blocked ficin

1 g of immobilized and blocked ficin was suspended in 10 mL of aminated BSA solution (10 mg/mL), in 50 mM carbonate buffer at pH 10.05 and room temperature. The suspension mixture was maintained 24 h under gentle stirring. The amount of immobilized BSA was quantified by the Bradford's method (the protein concentration of the initial solution is used as a reference). The biocatalyst was filtered and washed with excess of distilled water.

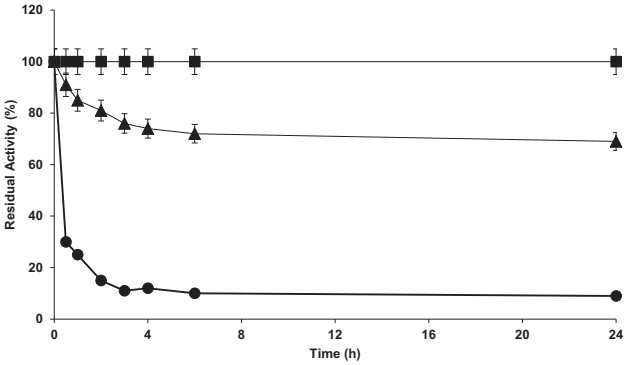

Fig. 1. Immobilization of ficin in glyoxyl-agarose at pH 10.05 and 25 °C. Other specifications are described in methods section. Squares: Reference; Triangles: immobilization suspension, Circles: supernatant of the immobilization conditions.

2.2.8. Modification of coimmobilized ficin-aminated BSA with aldehyde dextran

The ficin-aminated BSA biocatalyst was suspended in the solution of dextran aldehyde at pH 8 and left with continuous agitation for 48 h and room temperature. After 48 h, the mixture was reduced with $NaBH_4$ as described above and filtered and washed with excess of distilled water.

2.2.9. Coimmobilization of another layer of aminated BSA on coimmobilized ficin-aminated BSA-aldehyde dextran composite

The previous biocatalyst, without the reduction step, was filtered and washed with distilled water and suspended in a solution composed of aminated BSA in 50 mM sodium bicarbonate at pH 10 and room temperature, at a ratio 1:10 and using an aminated BSA concentration of 10 mg/ml. The mixture was left 24 h with gentle stirring. The amount of immobilized aminated BSA was quantified by the Bradford method (the protein concentration of the initial solution is used as a reference). Then, this biocatalyst mixture was reduced using 1 mg/mL of solid $NaBH_4$ for 30 min.. Afterwards, the biocatalyst was filtered and washed with excess of distilled water.

2.2.10. Blocked ficin biocatalysts incubation in β-mercaptoethanol

Reversibly inhibited biocatalysts of immobilized ficin were incubated in 50 mM sodium phosphate at pH 7 containing 1 M β-mercaptoethanol for 24 h (at a ratio 1/10) to recover the ficin activity. Finally, the samples were filtered, washed thoroughly with distilled water and stowed at 4 °C.

2.2.11. Inactivation of the different ficin preparations

Ficin-glyoxyl and ficin-glyoxyl modified with aminated BSA and dextran aldehyde were incubated at 64 °C and pH 7. At the defined times, samples were taken and the activity of the biocatalysts was determined using the casein and the BAPNA assays.

2.2.12. Operational stability of the blocked ficin-glyoxyl biocatalysts

The immobilized and modified ficin biocatalysts were incubated at 64 °C in 50 mM acetate buffer pH 5, 50 mM phosphate buffer pH 7 and 50 mM carbonate buffer pH 9. At the indicated times, samples were taken and the biocatalyst activity was quantified using BAPNA and casein as substrates. Activity was determined as described in enzymatic assay section.

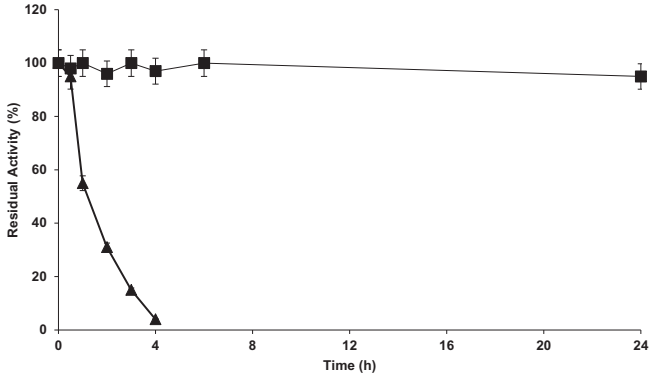

Fig. 2. Activity evolution of ficin-glyoxyl incubated in 5 mM 2PDS (squares) or 0.5 M urea/5 mM 2PDS (triangles). The experiments were performed as described in methods.

Table 1
Residual activity of ficin-glyoxyl biocatalysts incubated in different concentrations of urea in the presence or absence of 2PDS. Experiments were performed as described in methods.

2PDS	Concentration of urea,(M)				
	0.5	0.75	1	2	3
	Residual activity, (%)				
No	100	100	80	0	0
Yes	4	3	4	0	0

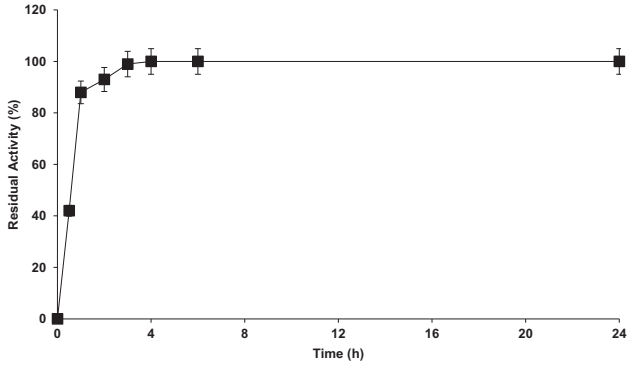

Fig. 3. Switch on of the 2PDS modified ficin-glyoxyl biocatalyst. 100 % of the activity corresponds to the activity of an unmodified biocatalyst. Experiments were performed as described in methods.

2.2.13. SDS-PAGE of the different ficin biocatalysts

The SDS-PAGE experiments were performed according to Laemmli [73] with minor changes. The free or immobilized enzymes were added to 4 % SDS (*w/v*) and 10 % mercaptoethanol (*v*/v) at 0.5 mg of protein/ml solution. These samples were boiled for 8 min and centrifuged to eliminate the support. 15 μL of the supernatants were injected in 12 % polyacrylamide gels, which was run at 100 V. The proteins were colored with Coomassie brilliant blue dye.

3. Results

3.1. Ficin immobilization and blocking of ficin

To have success in the planned strategy, the support should be full of ficin extract, that way a small excess of the enzyme was utilized in the immobilization [54]. Fig. 1 shows the immobilization course, the enzyme is rapidly immobilized, but a 10 % of the activity remained in the supernatant even after 24 h. This suggests that the full support area is

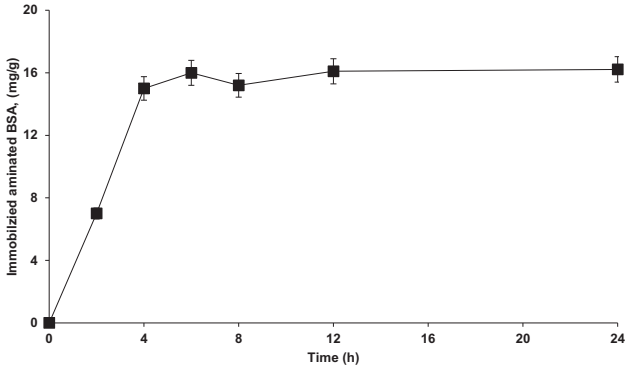

Fig. 4. Co-immobilization of aminated BSA on ficin-glyoxyl biocatalyst at pH 10 and 25 °C. Experiments were performed as described in methods.

Electrophoresis

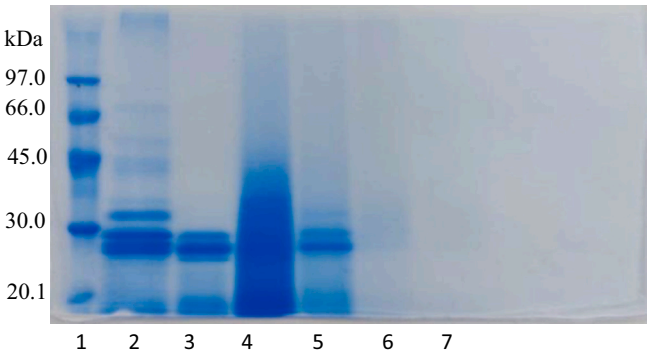

Fig. 5. SDS-PAGE analysis of different ficin preparations. Lane 1: low molecular weight protein standard from GE Healthcare. Lane 2: free ficin. Lane 3: no-reduced ficin-glyoxyl. Lane 4: no-reduced ficin-aminated BSA-glyoxyl. Lane 5: no-reduced ficin-aminated BSA-dextran-glyoxyl. Lane 6: reduced ficin-glyoxyl. Lane 9: reduced ficin- aminated BSA-dextran-aminated BSA-glyoxyl. Experiments were performed as described in Methods section.

coated with protein molecules. The suspension activity decreased (using the small synthetic substrate) to 70 % after 24 h [54] (Fig. 1).

Next, the biocatalysts were incubated with 2PDS to covalently (but reversibly) inhibit the ficin [57,59], with the objective of preventing the proteolysis of the proteins used to generate the steric hindrances. It has been reported that the simple incubation of the enzyme with this reagent was enough to fully block the enzyme activity, using the commercial ficin from Sigma [57,59]. However, now this incubation of the immobilized natural ficin had almost no effect on the immobilized enzyme activity (Fig. 2). It looks that the catalytic Cys in this natural immobilized enzyme was inaccessible to 2PDS. The free enzyme was neither inhibited by incubation with 2PDS. In order to increase the flexibility of the enzyme structure and that way, to be able to block the enzyme activity, we tried the modification of the biocatalysts in the presence of urea. Table 1 shows the results, using urea 0.5 or 0.75 M, the enzyme maintained the activity after 4 h in absence of 2PDS, and the activity versus BAPNA was nullified in the presence of this reagent. 2 and 3 M urea produced a serious impact on the ficin activity even in the absence of 2PDS, with no activity after 4 h. For this reason we decided to perform this inhibition step in the presence of 0.5 M urea.

Fig. 3 shows that this 2PDS blocked biocatalysts recovered the BAPNA activity by incubation in mercaptoethanol after 3 h of incubation. That way, we could switch off and switch on this biocatalyst activity, avoiding the undesired proteolysis of the "coating" protein that will be employed in further steps. The 2PDS blocked ficin-glyoxyl

Table 2
Residual activities of the different immobilized ficin biocatalyst versus BAPNA; casein and hemoglobin. Experiments were performed as described in methods.

Biocatalyst	Residual activity versus different substrates, (%)		
	Casein	Hemoglobin	BAPNA
Ficin-glyoxyl	100	100	100
BSA-ficin-glyoxyl	75.3	32.2	100
Dextran-BSA-ficin-glyoxyl	62.5	10.4	99
BSA-dextran-BSA-ficin-glyoxyl	61.5	3.1	97

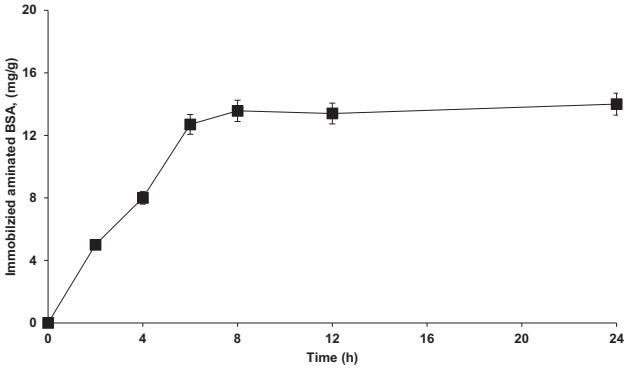

Fig. 6. Covalent immobilization of aminated BSA on dextran aldehyde-aminated BSA-ficin-glyoxyl biocatalyst at pH 10 and 25 °C. Experiments were performed as described in methods.

biocatalyst was the subject of further modifications.

We compared the initial activity of free ficin and immobilized ficin versus casein and hemoglobin. While using the free enzyme, in the standard conditions (55 °C), casein was hydrolyzed around 1.4-fold faster than hemoglobin, the immobilized enzyme hydrolyzed hemoglobin at 30 % the rate than hydrolyzed casein. This higher preference of the immobilized enzyme by casein can be caused by conformational chages in the enzyme or higher steric hindrances of the larger protein.

3.2. Generating steric hindrances to the ficin-glyoxyl biocatalyst

Next, aminated BSA was adsorbed on the ficin-glyoxyl biocatalyst. Fig. 4 shows the adsorption of the protein, about 16 mg BSA per gram of biocatalyst were absorbed. This was confirmed by SDS-PAGE of the biocatalyst (Fig. 5), where the BSA band can be clearly visualized (the protein is just physically adsorbed, that way, it can be visualized in the SDS-PAGE). Table 2 shows the effect of this modification on the enzyme activity versus the small BAPNA, and also versus casein and hemoglobin. The "blocked" biocatalyst fully maintained the activity versus BAPNA, decreased its activity versus casein to 75 % and the activity versus hemoglobin to 32 %. This suggested that the strategy can work, but that the BSA layer could leave too large "holes", enabling the entry of both large and small proteins. It cannot be fully discarded that the immobilized ficin extract modification by the aminated BSA can somehow affect the sequence ficin specificity, making that the modification affect more the activity versus hemoglobin than versus casein.

Next, the aminated BSA-ficin-glyoxyl biocatalyst was modified with dextran aldehyde (Table 2). This polymer is a random coil one, and there were some risks of modifying the ficin molecules, however, apparently, the direct modification of ficin was small or without effect on enzyme activity, as the activity versus BAPNA was almost fully maintained. The modification of the biocatalyst with this polymer was confirmed by SDS-PAGE (Fig. 5). Now, even without reduction, only a tiny band corresponding to ficin was observed, while BSA was not observed. This suggested that aldehyde dextran aldehyde was able to produce very stable bonds even before reduction, and that BSA is not released under SDS-

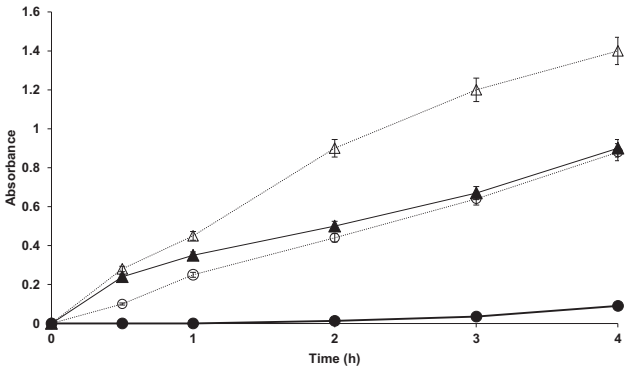

Fig. 7. Proteolysis reaction catalyzed by ficin-glyoxyl (empty symbols, pointed lines) or aminated BSA-dextran aldehyde-aminated BSA-ficin-glyoxyl biocatalysts (closed symbols, solid lines) using casein (triangles) or hemoglobin (circles) as substrates at 55 °C and pH 7. Other features are described in methods.

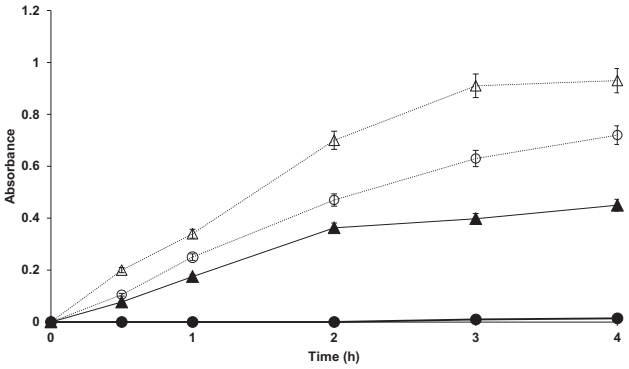

Fig. 8. Proteolysis reaction catalyzed by ficin-glyoxyl (empty symbols, pointed lines) or aminated BSA-dextran aldehyde-aminated BSA-ficin-glyoxyl biocatalysts (closed symbols, solid lines) using casein (triangles) or hemoglobin (circles) as substrates at 37 °C and pH 7. Other features are described in methods.

PAGE sample preparation. The activity versus casein decreased to more than 60 %, while versus hemoglobin decrease to 10 %. This result was quite positive, the specificity of the biocatalyst by casein compared to hemoglobin was increased by 6 folds, but still the biocatalyst presented a significant activity versus the large hemoglobin molecules. In this instance, considering that the direct ficin modification will be not possible, it is less likely that the ficin change in septicity may explain the results, although this cannot be fully discarded it is not possible to ensure the lack of ficin modification by the aldehyde dextran molecules.

To further increase the enzyme specificity, a new layer of aminated BSA was created over the aldehyde-dextran-BSA-ficin-glyoxyl biocatalyst (Fig. 6). The amount of immobilized BSA was slightly lower than in the first case (around 14 mg/g), as result of the smaller pore diameter and the subsequent reduction of the specific area. This biocatalyst still maintained around 95 % of the initial activity versus BAPNA, and almost 60 % versus casein, while the activity versus hemoglobin was clearly under 5 %. This means that the specificity of the ficin by casein was increased at least by a 12-fold factor by this protein multilayer, and that the activity versus hemoglobin was negligible. The reduction of the biocatalysts with sodium borohydride produced that not protein band could be observed in the SDS-PAGE experiments (Fig. 5). In this instance, it is even harder associate these changes to alteration of the ficin specificity by BSA modification, and it is quite unlikely that any of these BSA molecules can directly interact with the immobilized ficin extract.

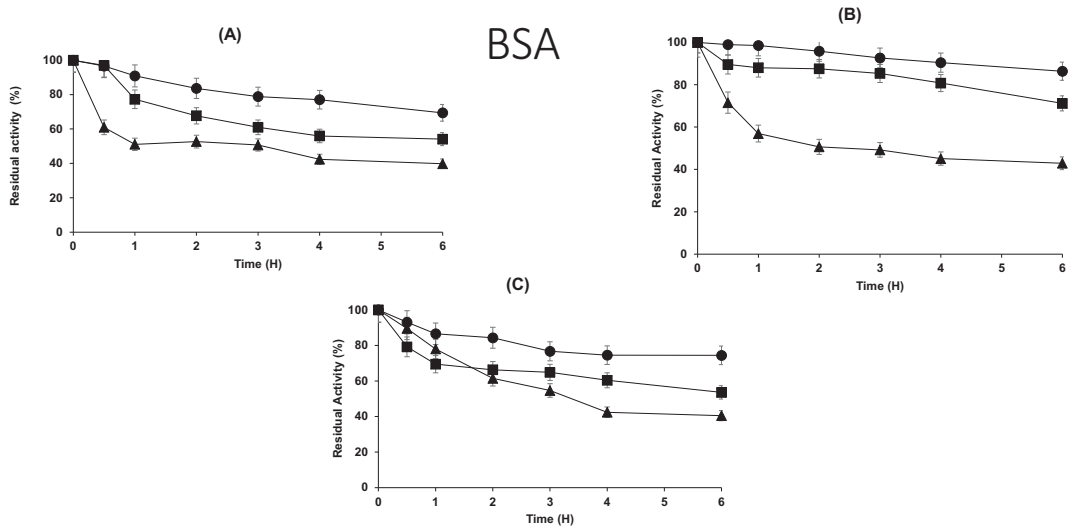

Fig. 9. Inactivation courses of different ficin using BAPNA as substrate at pH 5 (A), 7 (B) and 9 (C). 50 mM of the corresponding buffer was employed (see methods section) and the used Temperature was 64 °C. Other specifications can be found in Materials and Methods sections. Solid triangles: Ficin-glyoxyl; solid squares: Ficin-aminated BSA-glyoxyl; and solid circles: Ficin-aminated-BSA-dextran-aminated-BSA glyoxyl.

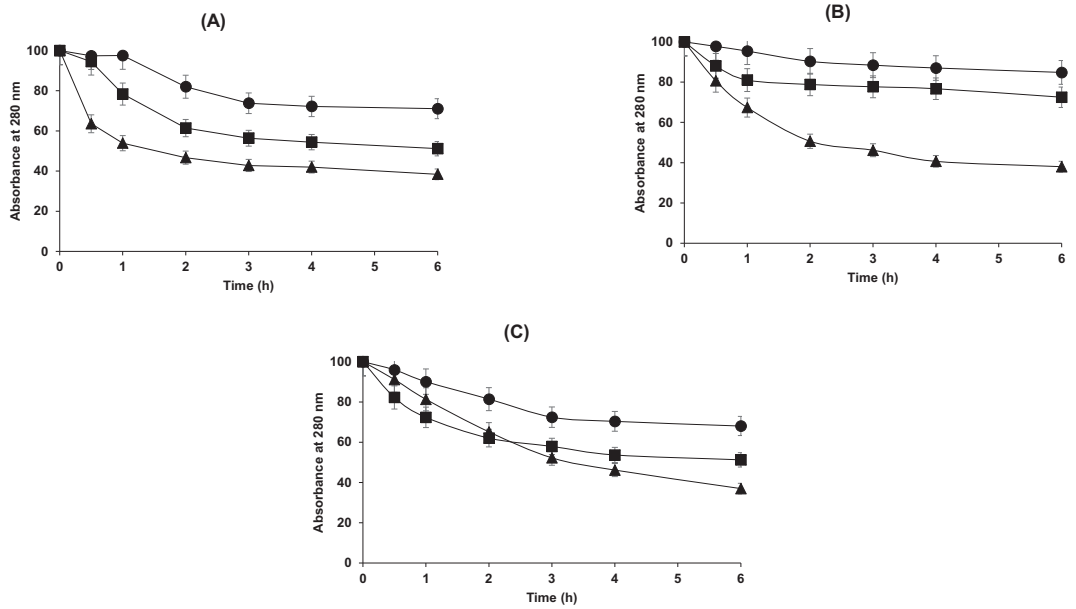

Fig. 10. Inactivation courses of different ficin using casein as substrate at pH 5 (A), 7 (B) and 9 (C). 50 mM of the corresponding buffer was employed (see methods section)and the used Temperature was 64 °C. Other specifications can be found in Materials and Methods sections. Solid triangles: Ficin-glyoxyl; solid squares: Ficin-aminated BSA-glyoxyl; and solid circles: Ficin-aminated-BSA-dextran-aminated-BSA glyoxyl.

3.3. Optimization of the specificity of the biocatalyst

The standard activity determination was performed at 55 °C, and under these conditions, we can expect that the flexibility of the coating layers and substrate proteins could be quite high. Even hemoglobin could partially dissociate under these conditions and the monomer become substrate of the enzyme [74–77]. Thus, the activities of the biocatalysts (blocked and unblocked) were examined using casein and hemoglobin at 37 °C. At this temperature, the activity of the blocked biocatalyst was 0 versus hemoglobin. To ensure the results, 4 h reaction courses were performed using both biocatalysts at 37 and 55 °C (Figs. 7 and 8). While at 55 °C some hydrolysis could be detected using hemoglobin and the double BSA coated biocatalyst (and accelerating with time, suggesting a role of the hemoglobin dissociation), at 37 °C the percentage of hemoglobin hydrolysis was negligible even after 4 h when using the double blocked biocatalyst. At both temperatures, casein could be readily proteolyzed by both biocatalysts, although the double blocked one was less active than the unblocked one. Very likely also casein molecules had more problems to reach the ficin active center by the increase in tortuosity in the pathway to the active center of the immobilized ficin molecules [46–51].

3.4. Effect of the modification of ficin-glyoxyl on the stability of the biocatalysts

Figs. 9 and 10 show the inactivation courses of the different ficin biocatalysts at pH 5, 7 and 9. At all pH values, the coating with aminated BSA presented a positive effect on enzyme stability, and the double coated biocatalyst gave the highest stabilities in all cases. Stabilization was more significant at pH 7 and less significant at pH 9. Similar

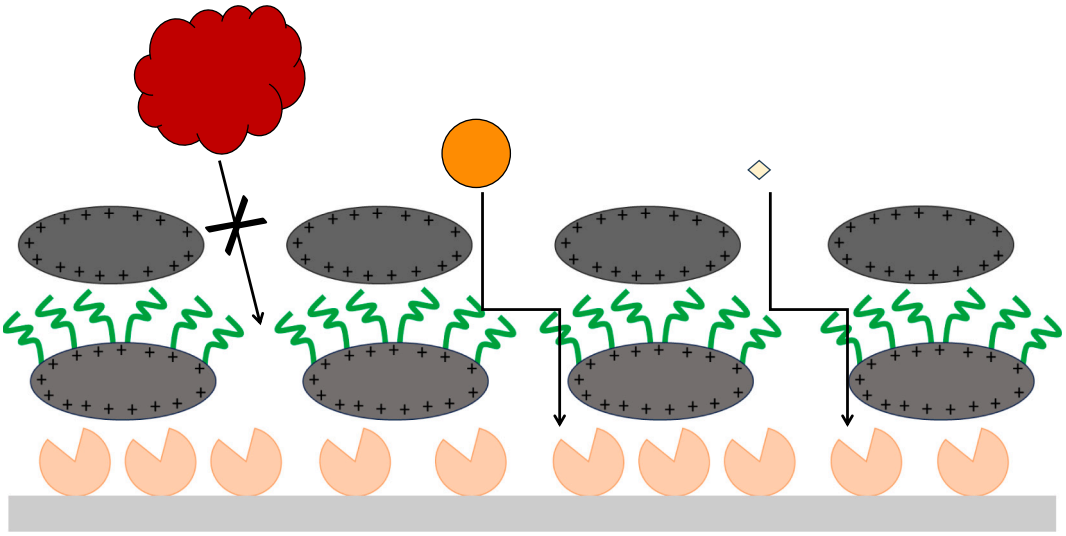

Fig. 11. Schematic representation of the developed strategy to generate steric hindrances to the immobilized ficin hydrolysis of large proteins.

qualitative results were obtained using BAPNA (Fig. 9) or casein (Fig. 10) as substrates.

The aminated-BSA-aldehyde dextran-BSA-ficin, glyoxyl biocatalyst could be reutilized for 10 4-h cycles of casein hydrolysis at pH 7 and 37 °C without any decrease on enzyme activity (not shown results). After this sixth reuse, the activity versus hemoglobin remained negligible.

4. Conclusion

The strategy developed in this paper to generate steric hindrances on immobilized proteases to increase its specificity versus small proteins has shown to be successful (Fig. 11). However, it has also shown that it is not as simple as generating steric hindrances for the immobilized enzymes, as the final biocatalysts still maintained around 50 % of the initial activity versus a protein of a similar size to the protease, this vale depending on the temperature. The switch off and switch on of the protease has permitted to prevent the proteolysis of the blocking proteins while they were in the immobilization process. The modification not only produces an increase of the biocatalyst towards small proteins, also produce an increment of the enzyme stability. The biocatalysts could be reused multiple cycles without affecting its activity. This could generate fragments of proteins that can make the reproducibility of the strategy complex. This new strategy opens up new possibilities in the area of biocatalysis, showing that we are still far from taking full advantage from the enzyme immobilization possibilities. To fully discard that the results shown in this paper may be, at least partially, consequence of changes on ficin sequence specificity, the use of a collection of proteins (that were good-enough substrates for ficin) bearing different sizes and sequences looks convenient.

CRediT authorship contribution statement

El Hocine Siar: Writing – original draft, Investigation, Formal analysis. **Pedro Abellanas-Perez:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Javier Rocha-Martin:** Writing – review & editing, Conceptualization. **Roberto Fernandez-Lafuente:** Writing – review & editing, Writing – original draft, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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