Journal of Biotechnology 395 (2024) 12–21

Contents lists available at ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

Designing tailor-made steric matters to improve the immobilized ficin specificity for small versus large proteins

El Hocine Siar [a,b], Pedro Abellanas-Perez [a], Roberto Morellon-Sterling [a], Juan M. Bolivar [c], Javier Rocha-Martin [d,*], Roberto Fernandez-Lafuente [a,*]

[a] Departamento de Biocatálisis, ICP-CSIC, Campus UAM-CSIC, Madrid 28049, Spain
[b] Agri-food Engineering Laboratory (GENIAAL), Institute of Food, Nutrition and Agri-Food Technologies (INATAA), University of Brothers Mentouri Constantine 1, Algeria
[c] FQPIMA group, Chemical and Materials Engineering Department, Faculty of Chemical Sciences, Complutense University of Madrid, Complutense Ave, Madrid 28040, Spain
[d] Department of Biochemistry and Molecular Biology, Faculty of Biology, Complutense University of Madrid, José Antonio Novais 12, Madrid 28040, Spain

ARTICLE INFO

Keywords:
Protease immobilization
Size specificity tuning
Steric hindrances

ABSTRACT

The development of strategies that can permit to adjust the size specificity of immobilized proteases by the generation of steric hindrances may enlarge its applicability. Using as a model ficin immobilized on glyoxyl agarose, two strategies were assayed to generate tailor made steric hindrances. First, ficin has been coimmobilized on supports coated with large proteins (hemoglobin or bovine serum albumin (BSA)). While coimmobilization of ficin with BSA presented no effect on the activity versus any of the assayed substrates, coimmobilization with hemoglobin permitted to improve the immobilized ficin specificity for casein versus hemoglobin, but still significant activity versus hemoglobin remained. Second, aldehyde-dextran has been employed to modify the immobilized ficin, trying to generate steric hindrances to avoid the entry of large proteins (hemoglobin) while enabling the entry of small ones (casein). This also increased the size specificity of ficin, but still did not suppress the activity versus hemoglobin. The combination of both strategies and the use of 37ºC during the proteolysis enabled to almost fully nullify the hydrolytic activity versus hemoglobin while preserving a high percentage of the activity versus casein. The modifications improved enzyme stability and the biocatalyst could be reused for 5 cycles without alteration of its properties.

1. Introduction

Enzyme immobilization was firstly developed to recover and reuse enzymes after their utilization as catalyst in a reaction (if they remained active), as initially enzymes were very expensive, and as water-soluble biocatalysts, their capture when used in free form is complex (Sheldon and van Pelt, 2013). Moreover, their immobilization permitted to benefit from the advantages of heterogeneous catalysis, regarding reactor design, control and simplification of product purification, and the decrease of wastes (Sheldon and van Pelt, 2013). Considering this compulsory necessity of enzyme immobilization, many researchers tried to take advantage of this step in the design of an industrial biocatalyst to solve other enzyme limitations (Di Cosimo et al., 2013; Garcia-Galan et al., 2011; Mateo et al., 2007; Rodrigues et al., 2013). The first focus was to improve enzyme stability by immobilization (Iyer and Ananthanarayan, 2008; Klibanov, 1979; Mateo et al., 2007). Although an inadequate immobilization protocol can even decrease enzyme stability, a suitable one can improve enzyme stability by increasing enzyme rigidity (e.g., via multipoint covalent attachment or fixation of more stable enzyme structures), preventing subunit dissociation of multimeric enzymes (via multi-subunit immobilization), generating positive enzyme environments (e.g., to promote the partition of hydrophilic or hydrophobic inactivating agents), etc. (Bolivar et al., 2022; Boudrant et al., 2020; Rodrigues et al., 2021). An adequate design of the support and/or immobilization conditions enables the purification of the enzyme during immobilization (in some instances requiring a genetic enzyme modification) (Barbosa et al., 2015; Lau et al., 2023; Qin et al., 2019; Sánchez-Otero et al., 2022; Wang et al., 2023b; Wang et al., 2023a; Zhou et al., 2020). Moreover, the interactions of the enzyme with the support surface and the fact that the enzyme is now in a confined

* Corresponding authors.
E-mail addresses: javrocha@ucm.es (J. Rocha-Martin), rfl@icp.csic.es (R. Fernandez-Lafuente).

https://doi.org/10.1016/j.jbiotec.2024.09.005
Received 29 July 2024; Received in revised form 4 September 2024; Accepted 6 September 2024
Available online 10 September 2024

space make it susceptible to becoming randomly distorted and submitted to steric and diffusional constraints (substrate, product, pH, etc.) (Garcia-Galan et al., 2011; Mateo et al., 2007; Rodrigues et al., 2013). This way, enzyme activity, selectivity, specificity, inhibition, etc. may be altered after immobilization (Rodrigues et al., 2013). Therefore, enzyme immobilization via different strategies may generate a collection of biocatalysts from the same enzyme with very different catalytic properties, increasing the possibilities of finding a biocatalyst with the desired performance in a specific process, even in a random way (Rodrigues et al., 2013).

One possibility that has been scarcely explored in the literature is the alteration of the specificity of the enzymes acting on macromolecular substrates by the size of the substrates via immobilization. This is the main topic of the current research effort. As model example, proteases will be utilized in this paper, as it may become interesting to have proteolytic biocatalysts able to only hydrolyze small proteins, leaving the large ones almost unmodified (Bilal et al., 2024; Tavano et al., 2018). This goal has been pursued by the generation of tailor-made steric hindrance on the immobilized enzyme. The objective is to difficulty the access of large proteins while enabling the access of other smaller proteins. This possibility of tailoring the enzyme size-specificity has been scarcely explored before, and it may permit to find new applications of the immobilized protease. For this purpose, we have proposed in this paper two different strategies to accomplish this specificity alteration of immobilized proteases towards small proteins regarding the large ones, both of them based on the tailored design generation of steric hindrances to the access of large proteins to the active center of immobilized proteases (Fig. 1).

The simplest strategy that we have assayed is the modification of the immobilized protease molecules with aldehyde dextran (Tacias-Pascacio et al., 2019). This modification will generate a flexible shell formed by a hydrophilic polymer with a random coil structure, and it may be expected that the steric problems generated for the access of protein molecules to the active center of the immobilized protease may be greater for large proteins than for small ones. The coating of supports with these polymers has proved to be able to prevent biomacromolecules multipoint interactions regarding one-point interaction with the treated supports (Fuentes et al., 2005; Mateo et al., 2001).

Another strategy will be the coimmobilization of the protease with another protein of a larger size that coats the support surface. If the surface of the support is full of this large protein and the protease molecules are immobilized in the spaces between large protein molecules, the protease will be in "pockets" formed by the surrounding larger proteins, and this should limit the access of proteins of a size similar to that of the large protein used to coat the support, while it should permit the entry of proteins and substrates of smaller size to a degree almost as if these large protein molecules are not near the protease molecules, just generating some minor diffusional limitations (the substrate may increase its tortuosity and the diffusional problems to reach the active center of the enzyme may be also enlarged). The combination of both approaches may be also studied if the individual strategies are not satisfactory enough. A similar methodology has been employed to prepare a chromatographic matrix where only small proteins can become immobilized on highly activated anion exchanger matrices (Bolivar et al., 2010).

As model immobilized protease, ficin (Abernethy and Leonardo, 1964; Morellon-Sterling et al., 2020) immobilized on glyoxyl agarose beads (Siar et al., 2020, 2017) has been selected. As this is a thiol protease, the possibility of switching the protease activity off and on allows preventing the undesired proteolysis of the immobilized large protein during protease immobilization, while recovering full enzyme activity after protease coimmobilization (Morellon-Sterling et al., 2022). This enzyme has diverse applications (Morellon-Sterling et al., 2020), and it has been shown that after immobilization on glyoxyl-agarose, its stability increases. However, the liability of the catalytic cysteine to oxidation causes stabilization to reach a plateau after some specific immobilization time, decreasing afterwards if inactivation is performed in the presence of oxygen (but not in anaerobic conditions) (Siar et al., 2023). As ficin has a molecular size of around 21 kDa (Azarkan et al., 2011; Devaraj et al., 2008; Englund et al., 1968; Porcelli, 1967), bovine serum albumin (BSA) (60 kDa) (Jahanban-Esfahlan et al., 2020; Lu et al., 2015; Majorek et al., 2012) and hemoglobin (a tetrameric protein of 64 kDa) (Fanelli et al., 1958; Perutz et al., 1960) were used as proteins to generate steric hindrances when coimmobilized with ficin. The selected substrates were a small synthetic substrate (benzoyl-arginine-p-nitroanilide (BAPNA) (Nakagawa et al., 1978; Oren and Galinski, 1994), hemoglobin and casein (around 23 kDa) (Dalgleish and Corredig, 2012; Horne, 2002, 1998).

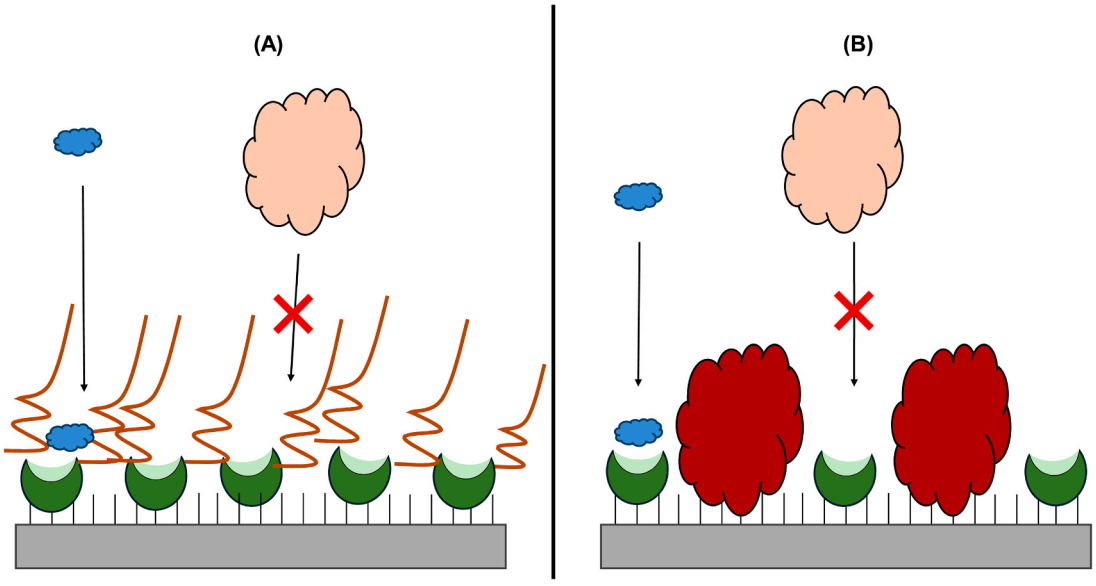

Fig. 1. Schematic representation of the two strategies proposed in this paper. First, the immobilized biocatalyst was modified with aldehyde-dextran (A), Second, ficin is coimmobilized in a support coated with a large protein (B).

2. Materials and methods

2.1. Materials

The preparation of glyoxyl agarose beads is described elsewhere (Grazu et al., 2005; Mateo et al., 2005) 2,2′-dipyridyldisulfide (2PDS) and hemoglobin were procured from Thermo-Fisher (Kandel, Germany). Agarose beads 4-BCL were acquired from Agarose Bead Technologies (ABT), (Alcobendas, Spain). BSA, casein, cysteine, 2-mercaptoethanol, sodium periodate, ethylenediaminetetraacetic acid (EDTA), 1-ethyl-3-(3--dimethylaminopropyl)-carbodiimide (EDC), ethylenediamine (EDA), glycidol, sodium borohydride and benzoyl-arginine-p-nitroanilide (BAPNA) were bought from Sigma-Aldrich (St. Louis, MO, USA). All other reagents were of analytical grade.

2.2. Methods

All experiments were performed in triplicate and the results are given as average and standard error.

2.2.1. Ficin preparation

Crude ficin extract was prepared as described by Siar et al., 2023 (Siar et al., 2023). After collection from Ficus carica L, the acquired latex was submitted to centrifugation (10,000 g for 15 min at 4ºC) and the supernatant was recovered and kept at – 20ºC until use, without any further treatment. The protein concentration was determined by Bradford method utilizing BSA to build the standard curve (Bradford, 1976).

2.2.2. Amination of hemoglobin and BSA with EDC and EDA

A mass of 2 g of proteins (hemoglobin or BSA) was added to 50 mL of a solution 1 M EDA. When the protein was dissolved and the pH adjusted to 4.57, solid EDC was added to the solution to reach a final concentration of 10 mM. After 90 minutes of gentle stirring at 25ºC, 100 % modification of all the exposed carboxylic groups was achieved (Hoare and Koshland, 1967). The aminated proteins were dialyzed (molecular weight cutoff of 10 kDa) 20–25ºC for 48 h. Dialysis was carried out in 3 L of 5 mM sodium phosphate pH 7, the dialysis buffer was changed after 2 h, 4 h, 8 h, 18 h, 24 h and 30 h.

2.2.3. Preparation of aldehyde dextran

Aldehyde dextran (6000. 40000 and 500000 Da) was prepared by complete oxidation with sodium periodate as previously described by Betancor et al., 2004 (Betancor et al., 2004). A 100 mL dextran solution was prepared in distilled water containing 3.33 g of dextran at 25 ∘C. When the dextran was fully dissolved, 8 g of sodium periodate were added under continuous stirring for 3 h, maintaining the temperature. The aldehyde-dextran solution was dialyzed against 50 volumes of distilled water, with 6 changes of dialysis buffer. The obtained aldehyde dextran was stored at 4ºC until use.

2.2.4. Enzymatic assays

Ficin biocatalysts activity was determined by using BAPNA, casein or hemoglobin as substrates.

In the BAPNA assay, first 43.5 mg of BAPNA were added to 1.0 mL of dimethyl sulfoxide. When it was dissolved, the solution was added to 99 mL of 0.1 M sodium phosphate pH 7.0, containing 5 mM of both, Cys and EDTA. Enzyme activity was determined by measuring the augmentation in absorbance produced by the liberation of p-nitroaniline at 405 nm at 25ºC (under these conditions, the ε was 8800). Activity is given in micromoles of produced p-nitroaniline per minute. The utilized spectrophotometer was a Jasco V 730 (Madrid, Spain) with control of temperature and magnetic stirring.

The method reported by Kunitz (Kunitz, 1947) was utilized to follow the hydrolysis of casein and hemoglobin, with slight modifications. Solutions of 1 % (w/v) casein or hemoglobin were prepared in 50 mM sodium phosphate at pH 7.0 containing 5 mM cysteine and 5 mM EDTA, and then it was heated to 55°C. 200 μL of biocatalyst suspensions or solutions was added to 1 mL of substrate solution, and the reaction mixture was incubated at the desired temperature for 15 min. The reaction was stopped by the addition of 2 mL of 10 % (w/v) trichloroacetic acid (TCA). This treatment yields the precipitation of the non-hydrolyzed protein, but the small peptides produced by the hydrolysis of proteins are maintained in soluble form. This suspension was centrifuged at 10,000 rpm for 5 min. The absorbance of the supernatant was measured at 280 nm. As reference, the enzyme was first inactivated by incubation in TCA, and then the protein substrate was added, following the same aforementioned protocol. Protease activity is given as 0.001 Absorbance increase per minute min−1. In some instances, the reaction time was prolonged to 6 h or the temperature was reduced to 37ºC.

2.2.5. Ficin modification with 2PDS

2PDS modification of ficin was carried out as described by Siar et al., 2023 with minor modifications (Morellon-Sterling et al., 2022; Siar et al., 2023). A solution of 5 mM 2 PDS in 5 mM sodium phosphate buffer containing 0.5 M urea and 10 % dimethyl-sulfoxide (DMSO) at pH 7 was prepared. The aim of this experiment was to reversibly modify the catalytic Cys residue (Morellon-Sterling et al., 2022; Siar et al., 2023). Then, ficin was added to a final concentration of 5 mg/mL and the suspension was submitted to constant agitation at 20–25ºC, and this reaction mixture was left for 4 h. Then, the reversibly inactivated ficin was dialyzed at 20–25ºC for 24 h using a dialysis membrane with a molecular weight cutoff of 10 kDa. Dialysis was performed in 3 L of 5 mM phosphate buffer pH 7, the dialysis buffer was changed after 2 h, 4 h, 8 h, 18 h and 24 h.

2.2.6. Immobilization of ficin extract on glyoxyl agarose beads

Immobilization of ficin (native or modified ficin with 2PDS) on glyoxyl-agarose beads (with or without proteins previously immobilized) was performed by adding 10 g of glyoxyl agarose to 100 mL of 1 mg/mL ficin extract solution prepared in 50 mM sodium carbonate at pH 10.05 at 20–25ºC under continuous stirring (Siar et al., 2023) for 3 h. Then, the suspension was reduced by adding solid NaBH4 to reach a concentration of 1 mg/mL. After gentle stirring for 30 min at 20–25ºC, the resulting ficin biocatalysts were washed with abundant distilled water (Boudrant et al., 2020).

2.2.7. Immobilization of BSA and hemoglobin on glyoxyl agarose beads

Immobilization of inert large proteins (aminated and non-aminated) on glyoxyl-agarose beads was carried out by adding 10 g of glyoxyl agarose to 100 mL of 20 mg/mL protein prepared in 50 mM sodium carbonate at pH 10.05 at 20–25ºC under continuous stirring for 24 h (a large excess of protein was used to coat the support with the large protein). Then, part of the suspension was taken and reduced by adding solid NaBH4 to reach a concentration of 1 mg/mL. After 30 min of gentle stirring at 20–25ºC, the immobilized protein preparations were washed with abundant distilled water. The other portion was utilized to coimmobilize ficin, as described below.

2.2.8. Coimmobilization of blocked ficin over the immobilized and aminated proteins

Immobilized aminated proteins preparations (without reduction of the aldehyde groups) were suspended in a blocked ficin solution (1 mg/mL), adding 1 g of immobilized protein matrix per 10 mL of blocked ficin in 50 mM carbonate buffer at pH 10.05. The immobilization mixture was left 24 h under gentle stirring at 20–25ºC. Next, part of the suspension was filtered and washed with excess distilled water. The remaining suspension was reduced by adding solid NaBH4 to reach a concentration of 1 mg/mL. After 30 min of gentle stirring at 20–25ºC, the biocatalysts were washed with abundant distilled water. In order to follow the course of immobilization, no blocked ficin was used.

2.2.9. Modification of ficin preparations with aldehyde dextran

Five grams of the ficin biocatalysts were suspended at 20–25ºC in 50 mL of dextran aldehyde at pH 8 and left with continuous agitation for 48 h. After this time, the mixture was reduced with NaBH4 as described above and filtered and washed with excess of distilled water.

2.2.10. Incubation of blocked ficin biocatalysts in β-mercaptoethanol to switch on ficin activity

Blocked immobilized ficin biocatalysts were suspended in 50 mM sodium phosphate at pH 7 containing 1 M of β-mercaptoethanol for 24 h (at a ratio 1/10) to recover the ficin activity. Then, the biocatalysts were washed with an excess of distilled water, filtered and stored at 4 °C. It was cross-checked that 2PDS-ficin and ficin immobilized on glyoxyl agarose exhibited identical activity after this reduction step, slightly higher than the glyoxyl immobilized ficin without this reduction step (Morellon-Sterling et al., 2022; Siar et al., 2023). That way, the blocked glyoxyl ficin biocatalysts further reduced with mercaptoethanol were used as reference in all cases, including the dextran modifications where the ficin is not 2PDS blocked (100 % of the activity).

2.2.11. Stress inactivation of the different ficin preparations

Different ficin biocatalysts were incubated at 64ºC in 50 mM of different buffers (sodium carbonate at pH 9, sodium acetate at pH 5 or sodium phosphate at pH 7). At the indicated times, samples were taken and the activity of the ficin biocatalysts was determined using both, the casein and the BAPNA assays described above.

2.2.12. Use of modified ficin-glyoxyl biocatalysts in casein and hemoglobin hydrolysis

The biocatalysts exhibiting the best performance regarding size specificity were used in casein and hemoglobin hydrolysis at 37ºC and 55ºC and pH 7. Hydrolysis was carried out in a reactor with continuous stirring and controlled temperature. 0.5 g of each biocatalyst was suspended in 10 mL of 1 % casein or hemoglobin (prepared in phosphate buffer 50 mM containing 5 mM cysteine and 5 mM EDTA) at pH 7. At the desired times, samples of 0.5 mL were taken (adding 1 mL of 10 % (w/v) TCA) and the soluble peptide concentration was determined after centrifugation as described above.

2.2.13. Reuse of ficin biocatalyst in the hydrolysis of casein

Five cycles of casein hydrolysis were performed at 37°C utilizing ficin biocatalysts. After 1 h of hydrolysis, the production of peptides was checked to quantify ficin activity, and the reaction was left to proceed for a total of 6 h. Then, the immobilized biocatalysts were filtered and washed with distilled water and employed in a new reaction cycle.

3. Results

3.1. Preparation of the biocatalysts

The aminated and non-aminated proteins were immobilized at pH 10.05. Hemoglobin immobilization was very slow, with low amounts of immobilized protein even after 24 h (Fig. 2). However, the immobilization of the aminated BSA and aminated hemoglobin was quite rapid (see Fig. 2 for hemoglobin). That way, no aminated proteins were discarded to coat the support surface. We left the immobilization process running for enough time to ensure that no new protein molecules could be immobilized, i.e. to ensure the full coating of the support surface with these large proteins. Next, ficin immobilization was performed on the support coated of these larger proteins; using a low loading, the space between the large immobilized protein molecules will be used for the enzyme immobilization (Bolivar et al., 2010). The immobilization of ficin on aminated-protein immobilized supports was slower than on naked supports, as it may be expected from the lower available space to immobilize ficin and higher diffusional limitations. Reversibly inhibited ficin and native enzyme were used in these experiments, although only the inhibited enzyme was used further. A representative example of the immobilization of ficin on blocked and naked glyoxyl agarose beads may be found in Fig. 3 (using the uninhibited enzyme). Immobilization was total in both cases with an expressed activity over 60 %. The Figure shows that, as previously reported, the immobilization of the large proteins left space between immobilized protein molecules enough to immobilize smaller molecules, in this instance ficin (Bolivar et al., 2010). The 2PDS-ficin biocatalyst and the unblocked ficin biocatalyst exhibited identical activity after incubation in mercaptoethanol.

Fig. 4 shows the SDS-analysis of the biocatalysts prepared with hemoglobin. The unreduced ficin-hemoglobin biocatalyst gave protein bands corresponding to ficin and hemoglobin, while after reduction, only hemoglobin bands were visible. The multimeric nature of

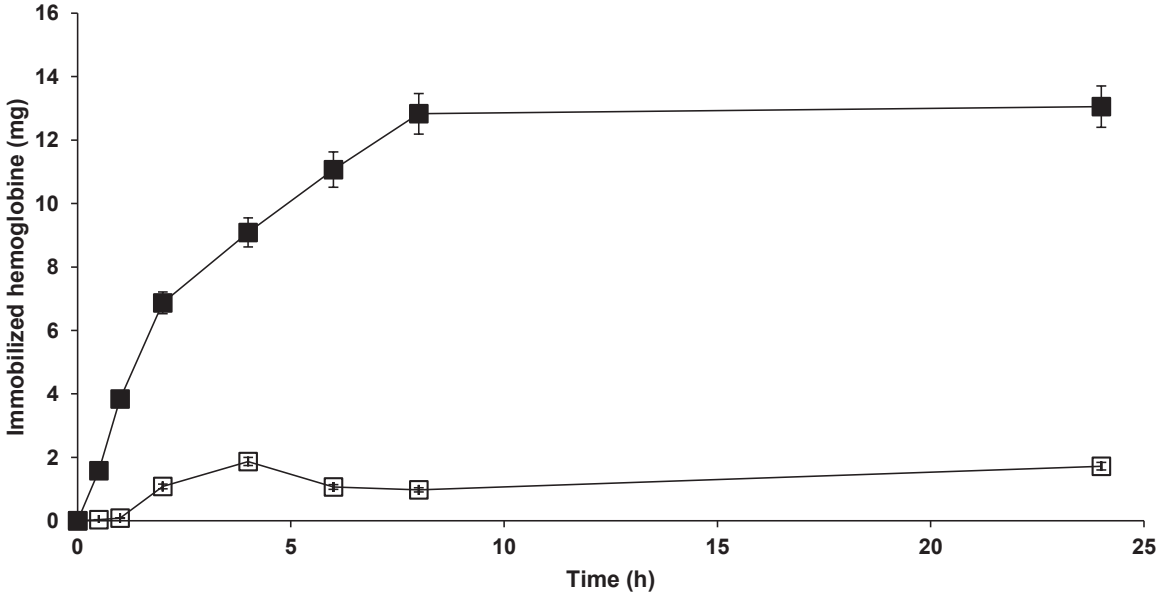

Fig. 2. Immobilization courses of different hemoglobin preparations in glyoxyl agarose beads. These experiments where performed at pH 10.05 at 20–25ºC. See methods section for further details. Solid squares: aminated hemoglobin; empty squares: natural hemoglobin.

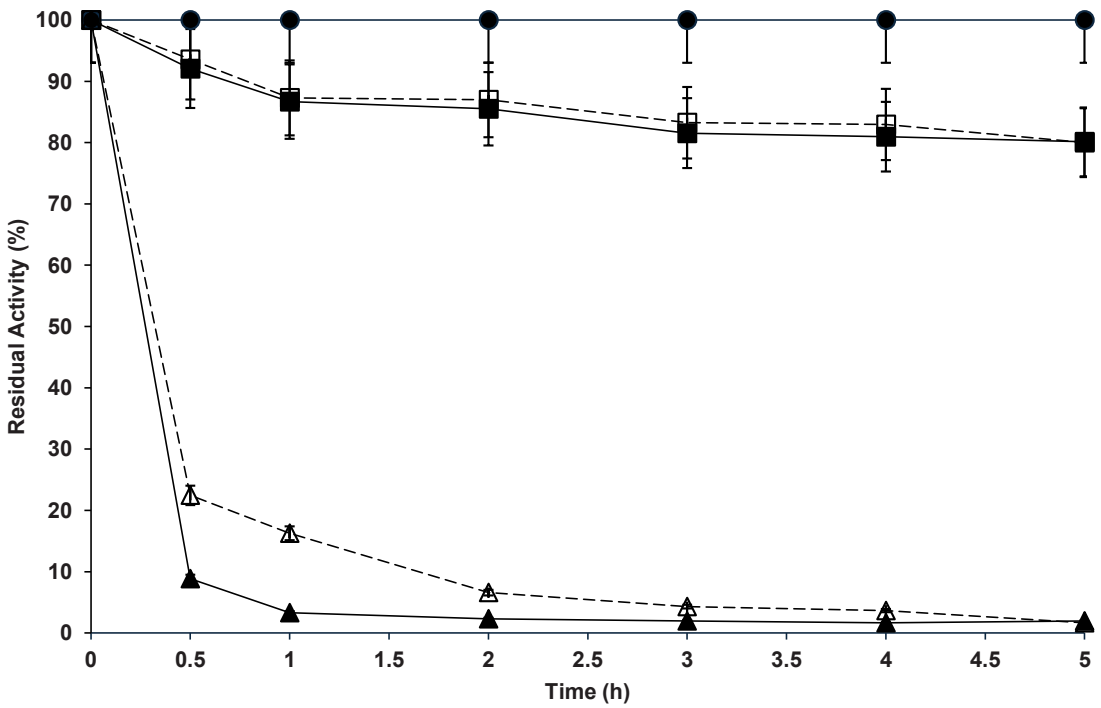

Fig. 3. Immobilization courses of ficin in different glyoxyl agarose supports at pH 10.05 at 20–25ºC. Further specifications can be found on the Methods section. Solid circles: free enzyme; Solid squares: ficin-glyoxyl suspension; Empty squares: ficin-hemoglobin-glyoxyl suspension; Solid triangles: ficin-glyoxyl supernatants; Empty triangles: ficin-hemoglobin-glyoxyl supernatants. Simultaneously, similar immobilizations were performed using reversibly inhibited ficin. Absolute activity of the free enzyme of the free enzyme at 37ºC was 1.38 units/mg using BAPNA, 0.92 units/mg using casein and 0.67 unit using hemoglobin.

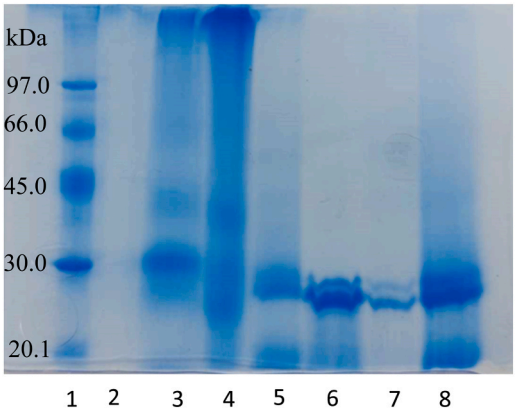

Fig. 4. SDS-PAGE analysis of different immobilized samples. Experiments were performed as described in Methods section. Lane 1: low molecular weight protein standard from GE Healthcare. Lane 2: reduced glyoxyl-hemoglobin-ficin-dextran. Lane 3: no reduced glyoxyl-hemoglobin-ficin dextran. Lane 4: no reduced glyoxyl-ficin-dextran. Lane 5: reduced glyoxyl-hemoglobin-ficin. Lane 6: no reduced glyoxyl-hemoglobin. Lane 7: reduced glyoxyl-hemoglobin. Lane 8: aminated hemoglobin.

hemoglobin explains the fact that even after reduction some protein could be visualized in the SDS-PAGE experiments, as not all proteins subunits are attached to the support (Balcão et al., 2001; Betancor et al., 2003; Bolivar et al., 2006). This was checked using a support bearing just the immobilized hemoglobin (Balcão et al., 2001; Betancor et al., 2003; Bolivar et al., 2006). The lack of proteins ficin bands after reduction confirmed that ficin was covalently immobilized on the support and not by physical interactions with the immobilized aminated proteins (very likely due to the high isoelectric point of the ficin) (Abernethy and Leonardo, 1964; Balcão et al., 2001; Betancor et al., 2003; Bolivar et al., 2006; Morellon-Sterling et al., 2020).

3.2. Activity of the different coimmobilized biocatalysts

The immobilized 2PDS-ficin biocatalysts were submitted to incubation with mercaptoethanol as described in the methods section to recover the enzyme activity. Next, the activity of the ficin biocatalyst prepared coimmobilizing ficin with different aminated inert proteins was determined using casein, hemoglobin and BAPNA at 55ºC and 37ºC. At 55ºC, the activity of the glyoxyl-ficin was around 27–33 % higher than at 37ºC versus all the substrates.

Table 1 shows the main results. The coimmobilization of ficin with aminated hemoglobin or BSA has no effect on the expressed activity of the biocatalyst versus BAPNA, the 3 biocatalysts presented identical activity at both 37 and 55ºC. This confirms that ficin did not suffer any changes due to the proximity of the large inert molecules. At 55ºC, the ficin activity versus casein suffered a slight decrease when coimmobilizing the enzyme with hemoglobin, while coimmobilization of ficin with BSA had no effect at all. This effect was far clearer at 37ºC when coimmobilizing ficin and hemoglobin to generate steric hindrances; in this case the activity of ficin coimmobilized with BSA remained unaltered versus casein also at this lower temperature.

Coimmobilizing ficin with hemoglobin decreased the proteolytic ficin activity using hemoglobin as substrate to 26 % at 55ºC and this effect was more significant at 37ºC. This effect of hemoglobin coimmobilization with ficin can be explained by the successful generation of steric problems to the entry of the free hemoglobin molecules towards the immobilized ficin molecules surrounded by large immobilized hemoglobin molecules, as they are immobilized on holes promoted by hemoglobin immobilization, and this should be lower than the size of a hemoglobin molecule (the support was fully coated with hemoglobin) (Bolivar et al., 2010). The effect of coimmobilized hemoglobin to the entry of hemoglobin substrate molecules to the coimmobilized ficin molecules is more relevant when the mobility of the substrate is lower; that way, it is more significant at 37ºC than at 55ºC. The effect of using glyoxyl-BSA to generate steric hindrances in coimmobilized ficin remained negligible at both, 55 or 37ºC. The lack of a coimmobilizing

Table 1
residual activity of different biocatalysts versus different substrates at 37ºC and 55ºC. Expressed in percentage of activity (%) relative to the activity of the ficin-glyoxyl biocatalyst. Additional information can be found in the methods section.

	BAPNA (37ºC)	BAPNA (55ºC)	Casein (37ºC)	Casein (55ºC)	Hemoglobin (37ºC)	Hemoglobin (55ºC)
Ficin-glyoxyl	100	100	100	100	100	100
Hem-ficin-glyoxyl	98±2	101±2	92±3	93±2	14±1	26±1
BSA-ficin-glyoxyl	99±2	98±2	102±2	102±3	103±2	105±2

effect of the protease with BSA can be explained because the BSA molecule presents an ellipsoidal form in one of the views of the enzyme structure (Fig. 5), and the immobilization very likely proceeds by the larger surface of the protein, leaving BSA molecules with a height regarding the support surface similar to ficin. This way, immobilized BSA did not generate steric problems to the entry of substrate hemoglobin to the immobilized ficin molecules active center.

That way, we have been able to significantly increase the specificity of the immobilized ficin biocatalyst towards large proteins (hemoglobin) when coimmobilizing the protease with hemoglobin, but even this biocatalyst still presented a significant activity versus the small one (casein).

3.3. Generation of steric hindrances by modifying immobilized ficin with aldehyde dextran

Next, we tried to generate steric hindrances to the entry of large proteins modifying the immobilized ficin with aldehyde dextran of different sizes (Fig. 1). Table 2 shows the effect of the modification with aldehyde dextran on the activity of the ficin biocatalysts. The effect versus BAPNA was negligible at both 37 and 55ºC. The modification was confirming by staining the modified biocatalyst with Schiff reagent, only the aldehyde dextran modified biocatalyst take the color (no the no modified biocatalysts or the naked agarose beads) Using casein, the activity decreased by around 40 % (a slightly higher decrease on casein lytic activity could be found using the smaller dextran). This could be explained because the smaller dextran can produce smaller “holes” in the shell surrounding the enzyme. These results mean that still a significant protease activity is maintained after the coating with aldehyde-dextran. This shows that the coating of enzymes with this flexible polymer may permit their interaction with molecules of a relatively large size, even rigid structures such as proteins. The decrease of casein lytic activity of ficin size-specificity is higher at 37 than at 55ºC, this may be explained by the mobility of both, the dextran shell and the substrate casein.

This reduction of proteolytic activity was more significant using large hemoglobin as substrate, with a similar trend with the temperature to the ones described above. The final balance is an increase of the ficin specificity by small proteins. However, even the dextran modified ficin biocatalyst with the lowest activity at 37ºC, still presented over 15 % of the activity versus hemoglobin compared to the initial one.

That way, again the strategy was partially successful, but some room for improvements was possible in our objective of nullifying the activity versus large proteins of the immobilized ficin while maintaining the activity versus small ones.

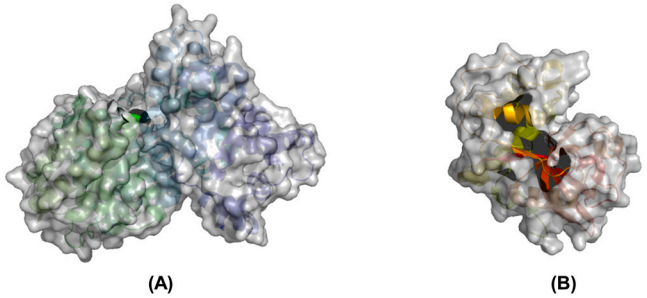

Fig. 5. 3D structure comparison of bovine serum albumin (A) (PDB: 4F5S) and ficin (B) (PDB: 4YYQ).

3.4. Modification with aldehyde dextran of ficin coimmobilized with hemoglobin

Trying to suppress the activity versus hemoglobin (large protein) while maintaining reasonable levels of activity versus casein (small protein), we combined the two strategies described above, modifying the hemoglobin-ficin biocatalyst with aldehyde dextran of different sizes. Fig. 4 shows that the unreduced biocatalyst released some protein after boiling in SDS, as reflected by some protein bands mainly of very large size (confirming the crosslinking induced by the aldehyde-dextran, and the high stability of the dextran-protein bonds, as they remain crosslinked even after the boiling step). This occurred also modifying just the immobilized hemoglobin with aldehyde dextran or ficin if the biocatalyst was not reduced. However, the reduced biocatalyst did not release any protein band, confirming that the dextran crosslinking step fully includes all hemoglobin subunits (Balcão et al., 2001; Betancor et al., 2003; Bolivar et al., 2006).

Table 3 shows the effects of the double modification on the ficin biocatalyst activity using the 3 substrates utilized in this paper. The activity versus BAPNA remained identical for the ficin and double ficin-modified biocatalyst at both 37 and 55ºC. Using casein, good levels of activity were maintained. In fact, the activity versus casein was higher than when modifying the ficin biocatalyst with aldehyde dextran (Table 2), perhaps now the aldehyde dextran mainly reacted with the aminated hemoglobin and did not reach the ficin molecules, preventing the undesired modification of ficin. At 55º, around 90 % of the activity versus casein was maintained; this value decreased to over 70 % at 37ºC. The differences between the effects of the size of the aldehyde dextran utilized in the modification on the activity versus casein were smaller than modifying ficin, being not very relevant. However, the activity versus hemoglobin was almost fully suppressed. At 55ºC, only between 2 % and 3 % of the proteolytic activity of ficin versus this large protein was maintained, while at 37ºC this was only around 1 %. This means that we have been able to almost fully nullify the activity versus hemoglobin of the immobilized ficin while maintaining reasonable levels of the activity versus casein.

3.5. Reaction curses in the hydrolysis of casein and hemoglobin using the initial ficin biocatalysts and the new biocatalyst

To ensure that this effect was maintained in the long term, Fig. 6 shows the reaction courses of hydrolysis of casein and hemoglobin at 55ºC for 6 h. While the hydrolysis of casein is only slightly affected, the hydrolysis rate of hemoglobin is significantly reduced; the increase in absorbance is very small even after 6 h of reaction using the double treated immobilized enzyme, compared to the results using the unmodified ficin or the results obtained using casein. The possibility of the immobilized and double modified ficin to be hydrolyzed into the dissociated subunits of hemoglobin cannot be discarded, as this temperature could favor hemoglobin dissociation (Bispo et al., 2007; Huang et al., 2013; Manning et al., 1996; Sever et al., 2021). At 37ºC (Fig. 7), the hydrolysis of hemoglobin is almost fully discarded using the double modified biocatalyst, and, although the hydrolysis of casein is reduced to some extent, the differences in the biocatalyst performance when

Table 2
residual activity of different ficin-glyoxyl biocatalysts modified with dextran sulfate of various sizes, versus different substrates at 37ºC and 55ºC. Expressed in percentage of activity (%) relative to the activity of the ficin-glyoxyl biocatalyst. Additional information can be found in the methods section.

	BAPNA (37ºC)	BAPNA (55ºC)	Casein (37ºC)	Casein (55ºC)	Hemoglobin (37ºC)	Hemoglobin (55ºC)
Ficin-dex 6000-glyoxyl	101±2	102±3	72 ±2	66±2	16±1	35±3
Ficin-dex 40000-glyoxyl	99±2	101±2	62±2	57±2	18±1	30±2
Ficin-dex 500000-glyoxyl	98±2	99±2	57±3	55±1	21±1	23±1

Table 3
Residual activity of different hemoglobin-ficin-glyoxyl biocatalysts modified with dextran sulfate of various sizes, versus different substrates at 37ºC and 55ºC. Expressed in percentage of activity (%) relative to the activity of the ficin-glyoxyl biocatalyst. Additional information can be found in the methods section. Activity of the glyoxyl agarose-ficin, corresponding to 100 % in each case, was 0.9 using BAPNA, 0.45 using casein and 0.42 using hemoglobin at 37ºC. At 55ºC the activities were 1.2, 0.55 and 0.5 respectively.

	BAPNA (37ºC)	**BAPNA (55ºC)**	**Casein (37ºC)**	**Casein (55ºC)**	**Hemoglobin (37ºC)**	**Hemoglobin (55ºC)**
Ficin-glyoxyl	100	100	100	100	100	100
Hem-ficin-glyoxyl	101±2	99±2	92±2	93±3	14±1	26±1
Hem-ficin-dex 6000-glyoxyl	99±2	96±3	72 ±2	91±2	1.82±0.21	3.39±0.24
Hem-ficin-dex 40000-glyoxyl	100	100	74±2	88±2	1.25±0.32	3.82±0.42
Hem-ficin-dex 500000-glyoxyl	100	100	74±1	89±2	1.53±0.25	2.67±0.33

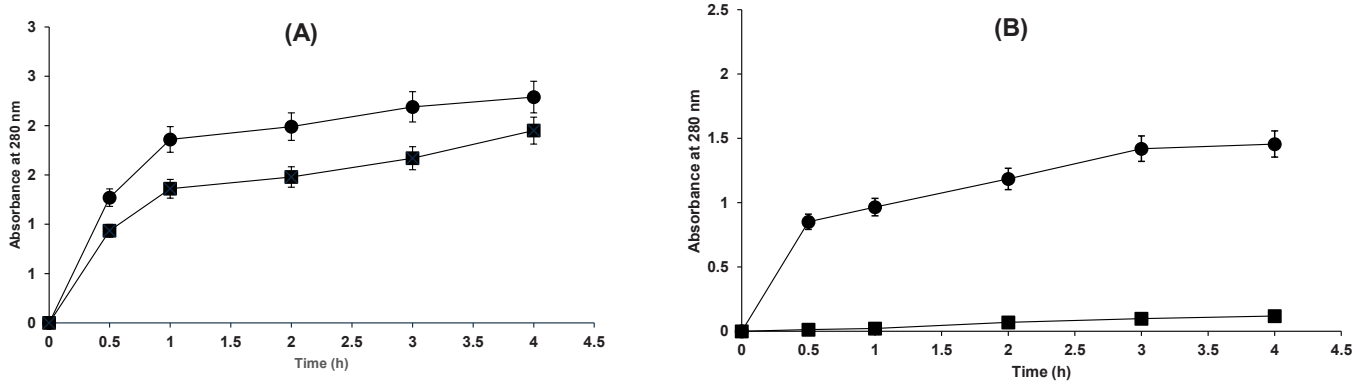

Fig. 6. Proteolytic reaction courses of casein (A) and hemoglobin (B) using different ficin biocatalysts. These experiments where performed at pH 7 and 55ºC. Further specifications can be found on the Methods section. Squares: ficin-hemoglobin-dextran-glyoxyl; solid circles: ficin-glyoxyl (all biocatalysts gave similar courses independently of the dextran size).

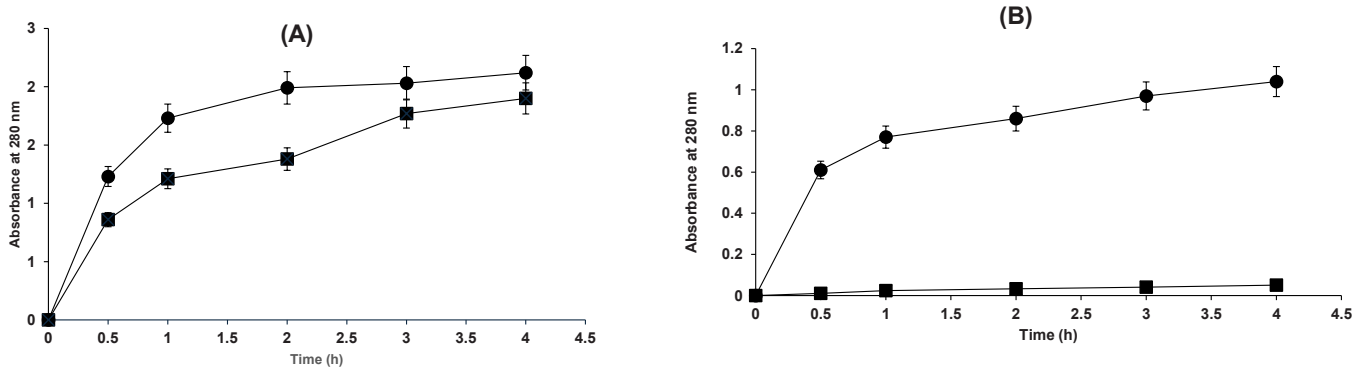

Fig. 7. Proteolytic reaction courses of casein (A) and hemoglobin (B) using different ficin biocatalysts**.** These experiments where performed at pH 7 and 37ºC. More specific details can be found on the Methods section Squares: ficin-hemoglobin-dextran-glyoxyl; solid circles: ficin-glyoxyl (all biocatalysts gave similar courses independently of the dextran size).

using casein and hemoglobin are very large. Hemoglobin hydrolysis is almost fully nullified by the double modified biocatalyst, while maintain good levels using casein, the casein/hemoglobin enzyme specificity increase by at least 70 folds.

3.6. Effect of the modification on the enzyme stability

Figs. 8 and 9 show the effect of the coimmobilized ficin treated with aldehyde dextran on its stability at different pH values, following the activity with BAPNA or casein, respectively. At all studied pH values (5, 7 and 9), the effect of the modifications on enzyme stability was positive, being the stabilization lower at alkaline pH and peaking at acidic pH.

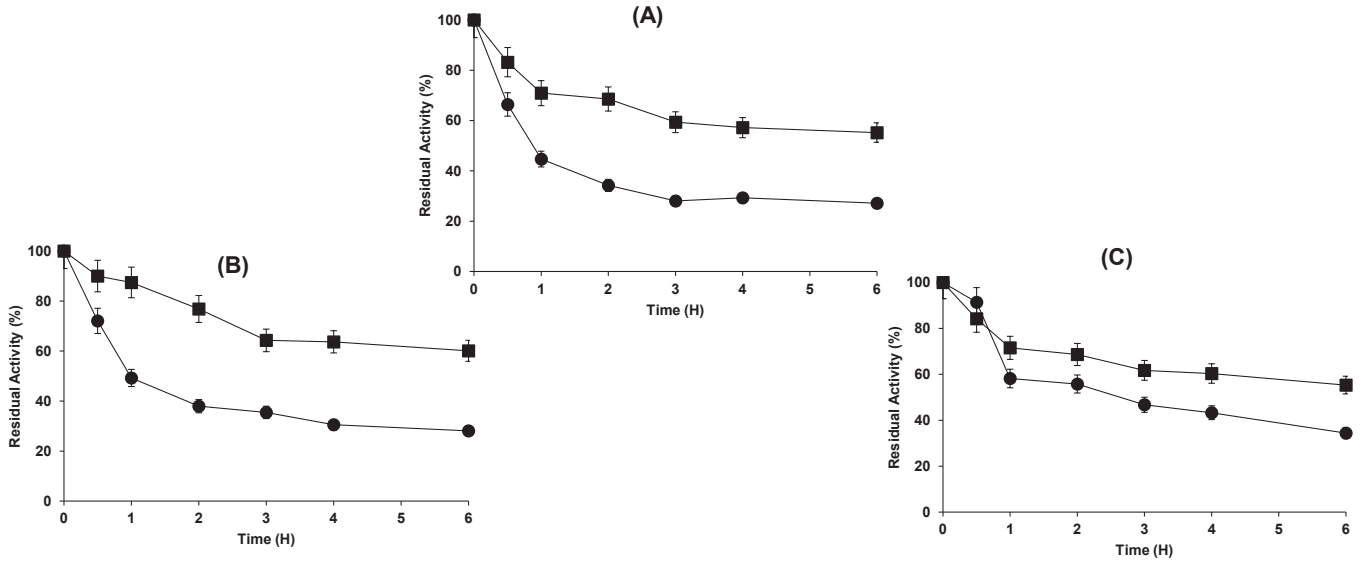

Fig. 8. Inactivation courses of different immobilized ficin biocatalysts at 64ºC and pH 5 (A), 7 (B) and 9 (C) using BAPNA as a substrate. More specifications can be found on the Methods section. Squares: ficin-hemoglobin-dextran-glyoxyl; solid circles: ficin-glyoxyl.

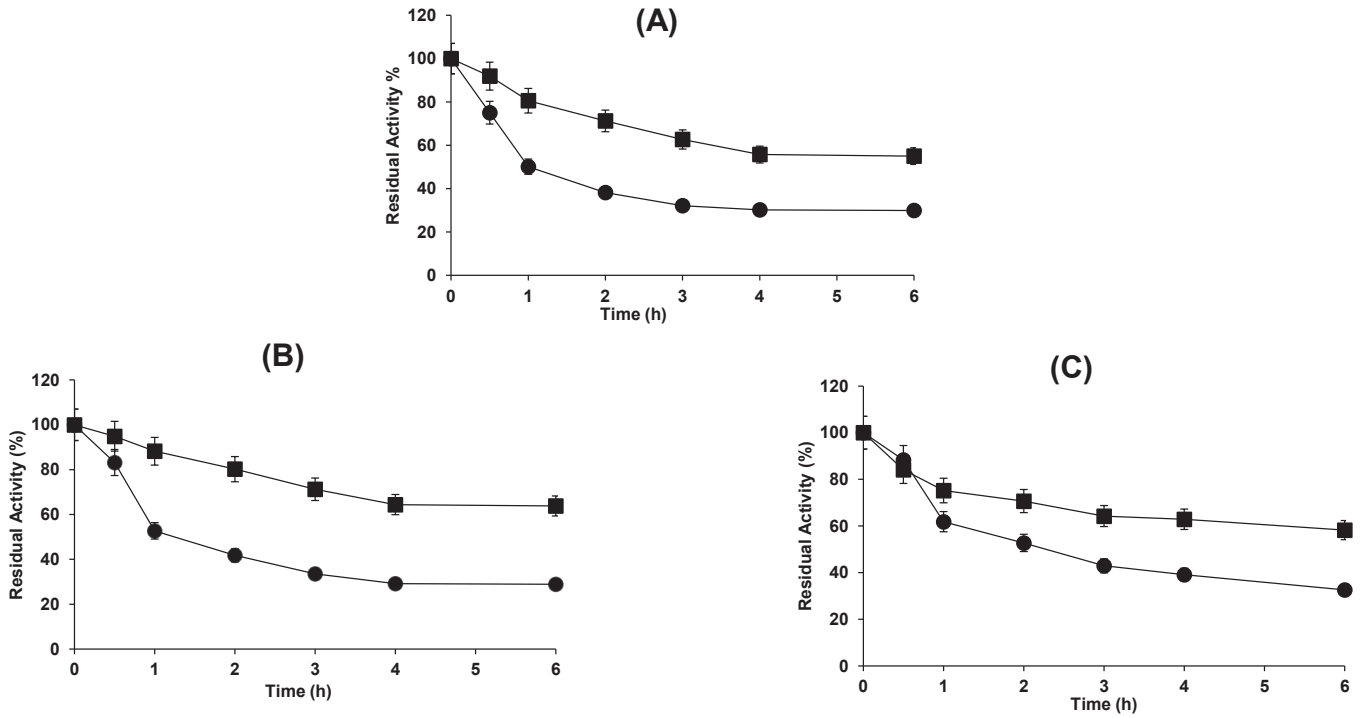

Fig. 9. Inactivation courses of the different immobilized ficin biocatalysts at 64ºC and pH 5 (A), 7 (B) and 9 (C) using casein as a substrate. More details can be found on the Methods section. Squares: ficin-hemoglobin-dextran-glyoxyl; solid circles: ficin-glyoxyl.

Although similar qualitative results were obtained with both substrates, using casein as substrate, the differences in the stabilization with the inactivation pH value decreased, becoming quite similar.

3.7. Operational stability of the biocatalyst

The double modified biocatalyst was utilized in 6 consecutive 6-hour reaction cycles at 37ºC, and Fig. 10 shows the results. The activity of the biocatalysts in hydrolysis of casein is not altered after these 6 cycles. Moreover, a similar experiment in the hydrolysis of hemoglobin maintained extremely low levels of hydrolysis during the 6 cycles (always in the range of 1 % of the activity of the initial glyoxyl-ficin biocatalyst (results not shown)).

4. Conclusions

The results reported in this paper show how the generation of tailor-made steric problems to the access of large versus the access of smaller macromolecules may be accomplished, achieving biocatalysts that mainly hydrolyze small proteins while becoming almost fully unable to modify large proteins. However, this only may be achieved after a careful design of the biocatalyst; it is not simple to nullify the activity

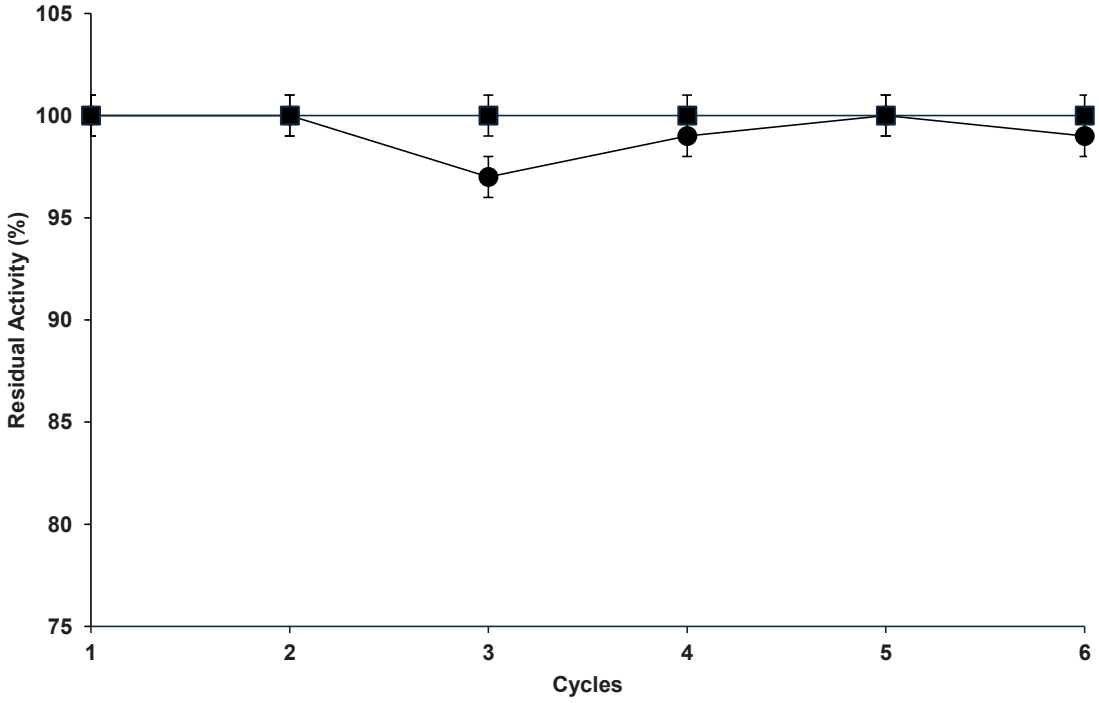

Fig. 10. Operational stability of different immobilized ficin biocatalysts. More specific details can be found on the Methods section. Squares: ficin-hemoglobin-dextran-glyoxyl; solid circles: ficin-glyoxyl.

versus large proteins, and even more complex to suppress the activity versus proteins of a size similar to that of the immobilized proteases. The generation of steric hindrances using only the coimmobilization with large proteins or the modification with aldehyde dextran enabled to increase enzyme specificity versus small proteins, but only the joint use of both strategies enables the almost complete nullification of the capacity of the immobilized ficin to hydrolyze large proteins. The positive effects of the modification make this strategy a promising one for the preparation of industrial biocatalysts intended to selectively hydrolyze small proteins.

CRediT authorship contribution statement

El Hocine Siar: Writing – original draft, Investigation, Formal analysis. **Pedro Abellanas-Perez:** Writing – review & editing, Writing – original draft, Investigation. **Roberto Morellon-Sterling:** Writing – original draft, Investigation. **Juan M. Bolivar:** Writing – original draft, Formal analysis. **Javier Rocha-Martin:** Writing – review & editing, Supervision, Formal analysis, Conceptualization. **Roberto Fernandez-Lafuente:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, 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.

Data Availability

No data was used for the research described in the article.

Acknowledgments

This research was funded by Ministerio de Ciencia e Innovación and Agencia Estatal de Investigación (Spanish Government) (PID2022–136535OB-I00). We gratefully recognize Prof. Ángel Berenguer-Murcia for his suggestions and help during the writing of this paper.

References

Abernethy, J.L., Leonardo, G.L., 1964. Ficin as a catalyst in organic syntheses. J. Chem. Educ. 41, 53–54. https://doi.org/10.1021/ed041p53.

Azarkan, M., Matagne, A., Wattiez, R., Bolle, L., Vandenameele, J., Baeyens-Volant, D., 2011. Selective and reversible thiol-pegylation, an effective approach for purification and characterization of five fully active ficin (iso)forms from Ficus carica latex. Phytochemistry 72, 1718–1731. https://doi.org/10.1016/j.phytochem.2011.05.009.

Balcão, V.M., Mateo, C., Fernández-Lafuente, R., Xavier Malcata, F., Guisán, J.M., 2001. Structural and functional stabilization of L-asparaginase via multisubunit immobilization onto highly activated supports. Biotechnol. Prog. 17, 537–542. https://doi.org/10.1021/bp000163r.

Barbosa, O., Ortiz, C., Berenguer-Murcia, Á., Torres, R., Rodrigues, R.C., Fernandez-Lafuente, R., 2015. Strategies for the one-step immobilization-purification of enzymes as industrial biocatalysts. Biotechnol. Adv. 33, 435–456. https://doi.org/10.1016/j.biotechadv.2015.03.006.

Betancor, L., Hidalgo, A., Fernández-Lorente, G., Mateo, C., Fernández-Lafuente, R., Guisan, J.M., 2003. Preparation of a stable biocatalyst of bovine liver catalase using immobilization and postimmobilization techniques. Biotechnol. Prog. 19, 763–767. https://doi.org/10.1021/bp025785m.

Betancor, L., López-Gallego, F., Hidalgo, A., Alonso-Morales, N., Fuentes, M., Fernández-Lafuente, R., Guisán, J.M., 2004. Prevention of interfacial inactivation of enzymes by coating the enzyme surface with dextran-aldehyde. J. Biotechnol. 110 https://doi.org/10.1016/j.jbiotec.2004.02.003.

Bilal, M., Qamar, S.A., Carballares, D., Berenguer-Murcia, Á., Fernandez-Lafuente, R., 2024. Proteases immobilized on nanomaterials for biocatalytic, environmental and biomedical applications: advantages and drawbacks. Biotechnol. Adv. 70, 108304 https://doi.org/10.1016/J.BIOTECHADV.2023.108304.

Bispo, J.A.C., Santos, J.L.R., Landini, G.F., Goncalves, J.M., Bonafe, C.F.S., 2007. pH dependence of the dissociation of multimeric hemoglobin probed by high hydrostatic pressure. Biophys. Chem. 125, 341–349. https://doi.org/10.1016/J.BPC.2006.09.009.

Bolivar, J.M., Batalla, P., Mateo, C., Carrascosa, A.V., Pessela, B.C., Guisán, J.M., 2010. Selective adsorption of small proteins on large-pore anion exchangers coated with medium size proteins. Colloids Surf. B Biointerfaces 78, 140–145. https://doi.org/10.1016/J.COLSURFB.2010.02.030.

Bolivar, J.M., Wilson, L., Ferrarotti, S.A., Guisán, J.M., Fernández-Lafuente, R., Mateo, C., 2006. Improvement of the stability of alcohol dehydrogenase by covalent immobilization on glyoxyl-agarose. J. Biotechnol. 125, 85–94. https://doi.org/10.1016/j.jbiotec.2006.01.028.

Bolivar, J.M., Woodley, J.M., Fernandez-Lafuente, R., 2022. Is enzyme immobilization a mature discipline? Some critical considerations to capitalize on the benefits of immobilization. Chem. Soc. Rev. 51, 6251–6290. https://doi.org/10.1039/D2CS00083K.

Boudrant, J., Woodley, J.M., Fernandez-Lafuente, R., 2020. Parameters necessary to define an immobilized enzyme preparation. Process Biochem. 90, 66–80. https://doi.org/10.1016/J.PROCBIO.2019.11.026.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem 72, 248–254. https://doi.org/10.1016/0003-2697(76)90527-3.

Dalgleish, D.G., Corredig, M., 2012. The structure of the casein micelle of milk and its changes during processing. Annu Rev. Food Sci. Technol. 3, 449–467. https://doi.org/10.1146/annurev-food-022811-101214.

Devaraj, K.B., Kumar, P.R., Prakash, V., 2008. Purification, characterization, and solvent-induced thermal stabilization of ficin from Ficus carica. J. Agric. Food Chem. 56, 11417–11423. https://doi.org/10.1021/jf802205a.

Di Cosimo, R., Mc Auliffe, J., Poulose, A.J., Bohlmann, G., 2013. Industrial use of immobilized enzymes. Chem. Soc. Rev. 42, 6437–6474. https://doi.org/10.1039/C3CS35506C.

Englund, P.T., King, T.P., Craig, L.C., Walti, A., 1968. Studies on Ficin. I. Its isolation and characterization. Biochemistry 7, 163–175. https://doi.org/10.1021/bi00841a021.

Fanelli, A.R., Antonini, E., Caputo, A., 1958. Studies on the structure of hemoglobin I. Physicochemical properties of human globin. Biochim Biophys. Acta 30, 608–615. https://doi.org/10.1016/0006-3002(58)90108-2.

Fuentes, M., Mateo, C., Guisán, J.M., Fernández-Lafuente, R., 2005. Preparation of inert magnetic nano-particles for the directed immobilization of antibodies. Biosens. Bioelectron. 20, 1380–1387. https://doi.org/10.1016/J.BIOS.2004.06.004.

Garcia-Galan, C., Berenguer-Murcia, Á., Fernandez-Lafuente, R., Rodrigues, R.C., 2011. Potential of different enzyme immobilization strategies to improve enzyme performance. Adv. Synth. Catal. 353, 2885–2904. https://doi.org/10.1002/ADSC.201100534.

Grazu, V., Guisan, J.M., Betancor, L., Fernandez-Lafuente, R., Lopez-Gallego, F., Montes, T., 2005. Glyoxyl agarose as a new chromatographic matrix. Enzym. Micro Technol. 38, 960–966. https://doi.org/10.1016/j.enzmictec.2005.08.034.

Hoare, D.G., Koshland Jr, D.E., 1967. A method for the quantitative modification and estimation of carboxylic acid groups in proteins. J. Biol. Chem. 242, 2447–2453.

Horne, D.S., 1998. Casein interactions: casting light on the black boxes, the structure in dairy products. Int Dairy J. 8, 171–177. https://doi.org/10.1016/S0958-6946(98)00040-5.

Horne, D.S., 2002. Casein structure, self-assembly and gelation. Curr. Opin. Colloid Interface Sci. 7, 456–461. https://doi.org/10.1016/S1359-0294(02)00082-1.

Huang, Y.X., Wu, Z.J., Huang, B.T., Luo, M., 2013. Pathway and mechanism of pH dependent human hemoglobin tetramer-dimer-monomer dissociations. PLoS One 8, e81708. https://doi.org/10.1371/JOURNAL.PONE.0081708.

Iyer, P.V., Ananthanarayan, L., 2008. Enzyme stability and stabilization-aqueous and non-aqueous environment. Process Biochem. 43, 1019–1032. https://doi.org/10.1016/J.PROCBIO.2008.06.004.

Jahanban-Esfahlan, A., Roufegarinejad, L., Jahanban-Esfahlan, R., Tabibiazar, M., Amarowicz, R., 2020. Latest developments in the detection and separation of bovine serum albumin using molecularly imprinted polymers. Talanta 207, 120317. https://doi.org/10.1016/J.TALANTA.2019.120317.

Klibanov, A.M., 1979. Enzyme stabilization by immobilization. Anal. Biochem 93, 1–25. https://doi.org/10.1016/S0003-2697(79)80110-4.

Kunitz, M., 1947. Crystalline soybean trypsin inhibitor: II. general properties. J. Gen. Physiol. 30, 291–310. https://doi.org/10.1085/jgp.30.4.291.

Lau, E.C.H.T., Dodds, K.C., McKenna, C., Cowan, R.M., Ganin, A.Y., Campopiano, D.J., Yiu, H.H.P., 2023. Direct purification and immobilization of his-tagged enzymes using unmodified nickel ferrite NiFe2O4 magnetic nanoparticles. Sci. Rep. 13, 1–10. https://doi.org/10.1038/s41598-023-48795-x.

Lu, R., Li, W.W., Katzir, A., Raichlin, Y., Yu, H.Q., Mizaikoff, B., 2015. Probing the secondary structure of bovine serum albumin during heat-induced denaturation using mid-infrared fiberoptic sensors. Analyst 140, 765–770. https://doi.org/10.1039/c4an01495b.

Majorek, K.A., Porebski, P.J., Dayal, A., Zimmerman, M.D., Jablonska, K., Stewart, A.J., Chruszcz, M., Minor, W., 2012. Structural and immunologic characterization of bovine, horse, and rabbit serum albumins. Mol. Immunol. 52, 174–182. https://doi.org/10.1016/J.MOLIMM.2012.05.011.

Manning, L.R., Jenkins, W.T., Hess, J.R., Vandegriff, K., Winslow, R.M., Manning, J.M., 1996. Subunit dissociations in natural and recombinant hemoglobins. Protein Sci. 5, 775–781. https://doi.org/10.1002/PRO.5560050423.

Mateo, C., Abian, O., Bernedo, M., Cuenca, E., Fuentes, M., Fernandez-Lorente, G., Palomo, J.M., Grazu, V., Pessela, B.C.C., Giacomini, C., Irazoqui, G., Villarino, A., Ovsejevi, K., Batista-Viera, F., Fernandez-Lafuente, R., Guisán, J.M., 2005. Some special features of glyoxyl supports to immobilize proteins. Enzym. Micro Technol. 37, 456–462. https://doi.org/10.1016/J.ENZMICTEC.2005.03.020.

Mateo, C., Fernandez-Lorente, G., Pessela, B.C.C., Vian, A., Carrascosa, A.V., Garcia, J.L., Fernandez-Lafuente, R., Guisan, J.M., 2001. Affinity chromatography of polyhistidine tagged enzymes - New dextran-coated immobilized metal ion affinity chromatography matrices for prevention of undesired multipoint adsorptions. J. Chromatogr. A 915, 97–106. https://doi.org/10.1016/S0021-9673(01)00626-4.

Mateo, C., Palomo, J.M., Fernandez-Lorente, G., Guisan, J.M., Fernandez-Lafuente, R., 2007. Improvement of enzyme activity, stability and selectivity via immobilization techniques. Enzym. Micro Technol. 40, 1451–1463. https://doi.org/10.1016/J.ENZMICTEC.2007.01.018.

Morellon-Sterling, R., Bolivar, J.M., Fernandez-Lafuente, R., 2022. Switch off/switch on of a cysteinyl protease as a way to preserve the active catalytic group by modification with a reversible covalent thiol modifier: Immobilization of ficin on vinyl-sulfone activated supports. Int J. Biol. Macromol. 220, 1155–1162. https://doi.org/10.1016/j.ijbiomac.2022.08.155.

Morellon-Sterling, R., El-Siar, H., Tavano, O.L., Berenguer-Murcia, Á., Fernández-Lafuente, R., 2020. Ficin: a protease extract with relevance in biotechnology and biocatalysis. Int J. Biol. Macromol. 162, 394–404. https://doi.org/10.1016/j.ijbiomac.2020.06.144.

Nakagawa, H., Shuto, K., Tsurufuji, S., 1978. An improved method for determining the activity of benzoyl-arginine p-nitroanilide-hydrolyzing enzyme in crude tissue extracts. Anal. Biochem 87, 257–262. https://doi.org/10.1016/0003-2697(78)90592-4.

Oren, A., Galinski, E.A., 1994. Hydrolysis of N'-benzoyl-arginine-p-nitroanilide stereoisomers as a phenotypic test: a study of gram-positive halotolerant bacteria. Syst. Appl. Microbiol 17, 7–10. https://doi.org/10.1016/S0723-2020(11)80024-X.

Perutz, M.F., Rossmann, M.G., Cullis, A.F., Muirhead, H., Will, G., North, A.C., 1960. Structure of hæmoglobin: a three-dimensional fourier synthesis at 5.5-Å. resolution, obtained by X-ray analysis. Nature 185, 416–422.

Porcelli, G., 1967. Investigations on ficin. III. purification of ficin by gel filtration and the characterization of other protein fractions of ficuslatex. J. Chromatogr. A 28, 44–48. https://doi.org/10.1016/s0021-9673(01)85926-4.

Qin, Z., Lin, S., Qiu, Y., Chen, Q., Zhang, Y., Zhou, J., Zhao, L., 2019. One-step immobilization-purification of enzymes by carbohydrate-binding module family 56 tag fusion. Food Chem. 299 https://doi.org/10.1016/J.FOODCHEM.2019.125037.

Rodrigues, R.C., Berenguer-Murcia, Á., Carballares, D., Morellon-Sterling, R., Fernandez-Lafuente, R., 2021. Stabilization of enzymes via immobilization: multipoint covalent attachment and other stabilization strategies. Biotechnol. Adv. 52, 107821 https://doi.org/10.1016/J.BIOTECHADV.2021.107821.

Rodrigues, R.C., Ortiz, C., Berenguer-Murcia, Á., Torres, R., Fernández-Lafuente, R., 2013. Modifying enzyme activity and selectivity by immobilization. Chem. Soc. Rev. 42, 6290–6307. https://doi.org/10.1039/C2CS35231A.

Sánchez-Otero, M.G., Quintana-Castro, R., Rojas-Vázquez, A.S., Badillo-Zeferino, G.L., Mondragón-Vázquez, K., Espinosa-Luna, G., Kumar Nadda, A., Oliart-Ros, R.M., 2022. Polypropylene as a selective support for the immobilization of lipolytic enzymes: hyper-activation, purification and biotechnological applications. J. Chem. Technol. Biotechnol. 97, 436–445. https://doi.org/10.1002/jctb.6876.

Sever, A.I.M., Yin, V., Konermann, L., 2021. Interrogating the quaternary structure of noncanonical hemoglobin complexes by electrospray mass spectrometry and collision-induced dissociation. J. Am. Soc. Mass Spectrom. 32, 270–280. https://doi.org/10.1021/JASMS.0C00320/SUPPL_FILE/JS0C00320_SI_001.PDF.

Sheldon, R.A., van Pelt, S., 2013. Enzyme immobilisation in biocatalysis: why, what and how. Chem. Soc. Rev. 42, 6223–6235. https://doi.org/10.1039/C3CS60075K.

Siar, E.-H., Morellon-Sterling, R., Carballares, D., Rocha-Martin, J., Barbosa, O., Bolivar, J.M., Fernandez-Lafuente, R., 2023. Glyoxyl-ficin: an example where a more intense multipoint covalent attachment may decrease enzyme stability. Process Biochem. 132, 289–296. https://doi.org/10.1016/j.procbio.2023.07.029.

Siar, E.-H., Morellon-Sterling, R., Zidoune, M.N., Fernandez-Lafuente, R., 2020. Use of glyoxyl-agarose immobilized ficin extract in milk coagulation: unexpected importance of the ficin loading on the biocatalysts. Int J. Biol. Macromol. 144, 419–426. https://doi.org/10.1016/j.ijbiomac.2019.12.140.

Siar, E.-H., Zaak, H., Kornecki, J.F., Zidoune, M.N., Barbosa, O., Fernandez-Lafuente, R., 2017. Stabilization of ficin extract by immobilization on glyoxyl agarose. Preliminary characterization of the biocatalyst performance in hydrolysis of proteins. Process Biochem. 58, 98–104. https://doi.org/10.1016/j.procbio.2017.04.009.

Tacias-Pascacio, V.G., Ortiz, C., Rueda, N., Berenguer-Murcia, Á., Acosta, N., Aranaz, I., Civera, C., Fernandez-Lafuente, R., Alcántara, A.R., 2019. Dextran aldehyde in biocatalysis: more than a mere immobilization system. Catalysts 9. https://doi.org/10.3390/catal9070622.

Tavano, O.L., Berenguer-Murcia, A., Secundo, F., Fernandez-Lafuente, R., 2018. Biotechnological applications of proteases in food technology. Compr. Rev. Food Sci. Food Saf. 17, 412–436. https://doi.org/10.1111/1541-4337.12326.

Wang, L., Lan, H., Guan, W., Han, J., Liu, Y., Wang, Yu, Mao, Y., Wang, Yun, 2023. One-step purification of target enzymes using interaction- and structure-based design of aptamer-affinity responsive polymers: selective immobilization and enhanced stability. Sep Purif. Technol. 307, 122758 https://doi.org/10.1016/J.SEPPUR.2022.122758.

Wang, K., Zhao, L., Li, T., Wang, Q., Ding, Z., Dong, W., 2023. Selective immobilization of his-tagged enzyme on Ni-chelated ion exchange resin and its application in protein purification. Int J. Mol. Sci. 24 https://doi.org/10.3390/ijms24043864.

Zhou, J., Chen, J., Zhuang, N., Zhang, A., Chen, K., Xu, N., Xin, F., Zhang, W., Dong, W., Jiang, M., 2020. Immobilization and purification of enzymes with the novel affinity tag ChBD-AB from chitinolyticbacter meiyuanensis SYBC-H1. Front Bioeng. Biotechnol. 8, 1–10. https://doi.org/10.3389/fbioe.2020.00579.