

## Article

# Immobilization/Stabilization of Ficin Extract on Glutaraldehyde-Activated Agarose Beads. Variables That Control the Final Stability and Activity in Protein Hydrolyses

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Received: 12 March 2018; Accepted: 2 April 2018; Published: 6 April 2018



**Abstract:** Ficin extract has been immobilized on different 4% aminated-agarose beads. Using just ion exchange, immobilization yield was poor and expressed activity did not surpass 10% of the offered enzyme, with no significant effects on enzyme stability. The treatment with glutaraldehyde of this ionically exchanged enzyme produced an almost full enzyme inactivation. Using aminated supports activated with glutaraldehyde, immobilization was optimal at pH 7 (at pH 5 immobilization yield was 80%, while at pH 9, the immobilized enzyme became inactivated). At pH 7, full immobilization was accomplished maintaining 40% activity versus a small synthetic substrate and 30% versus casein. Ficin stabilization upon immobilization could be observed but it depended on the inactivation pH and the substrate employed, suggesting the complexity of the mechanism of inactivation of the immobilized enzyme. The maximum enzyme loading on the support was determined to be around 70 mg/g. The loading has no significant effect on the enzyme stability or enzyme activity using the synthetic substrate but it had a significant effect on the activity using casein; the biocatalysts activity greatly decreased using more than 30 mg/g, suggesting that the near presence of other immobilized enzyme molecules may generate some steric hindrances for the casein hydrolysis.

**Keywords:** immobilization using glutaraldehyde; versatility of glutaraldehyde; steric problems in enzyme activity; effect of loading on enzyme activity

## 1. Introduction

Proteases are among the first enzymes used by humans in biotechnological food processing [1,2]. Nowadays, proteases are used in many different areas, for example in the production of active peptides from inexpensive proteins, to improve the organoleptic or functional properties of foods [3], although perhaps its main use is as detergent components [4–7]. In some instances, a chemical alternative exists [8] but proteases have clear advantages, as the process will be more specific, avoiding the production of by-products or destruction of some amino acids [9]. Among the most used proteases of vegetal origin, the latex of the fig tree (based in ficin) should be mentioned. It has been employed in the brewing [9], pharmaceutical [10] and in cheese making [11,12] industries. It has also been used

for bioactive peptides production [13,14] and meat tenderization [15,16]. Ficin is considered to give a more reproducible hydrolysis map and it has been employed in the generation of antibodies via specific hydrolysis of some peptide bonds, [17–19]. Four isoforms of ficin have been described (A, B, C and D) and crystallized, resolving their structure [20,21]. Ficin isoforms are glycoproteins and present a high sequence similarity with bromelaine [22].

The immobilization of an enzyme may facilitate enzyme reuse and that way, the economic feasibility of the process [23–26]. Moreover, immobilization may be used to improve other enzyme limitations, like stability, activity, resistance to chemicals or inhibitors, even purity [27–29]. In the case of proteases to be used in protein hydrolysis, there are specific problems that must be considered: the enzyme must have the active centre properly oriented; otherwise the enzyme will be inactive [1]. Only when the active centre is fully oriented to the medium, the protease will be active under any loading; if the active centre is not “perfectly” oriented, the fully loaded biocatalyst may become fully inactive versus proteins larger than the immobilized protease molecule [30]. This steric problem will be coupled to the standard diffusional limitations, even if the active centre is fully intact [31] or a non-porous nanoparticle is utilized [32]. However, due to the multiple uses of proteases, this should not discard the biocatalysts for other uses, for example synthesis of peptide bonds [33–35]. Ficin has been immobilized in just some few examples [36–40].

In this sense, immobilization of ficin using the glutaraldehyde chemistry may benefit from the versatility of the system [41]. In most cases, glutaraldehyde is used to activate supports containing primary amino groups. This way, the glutaraldehyde activated support becomes a heterofunctional one, with the range of opportunities that this may open. Thus, the enzyme may be directly immobilized at high ionic strength via a covalent reaction using glutaraldehyde pre-activated supports, or may be first ionically exchanged [42–44]. That way, using a support with the amino groups modified with glutaraldehyde, the enzymes may be ionically exchanged at low ionic strength and later a covalent reaction between the immobilized enzyme molecules and the nearby glutaraldehyde groups may take place, or using high ionic strength, the ionic adsorption will be prevented, forcing the covalent attachment as first event of the immobilization. Using just the aminated support, the ionic exchange will permit to immobilize the enzyme, later the treatment with glutaraldehyde may permit to establish support-enzyme bonds. In this case, together with enzyme-support reactions, modification of the overall enzyme surface occurs (one point modification, inter or intramolecular crosslinking) and this may have positive, negative or neutral effects on enzyme performance [41]. The possibilities of altering the enzyme orientation regarding the support surface and that way, of increasing the possibility of yielding immobilized enzyme preparations with different properties, may be enlarged considering that the conditions where the ion exchange is performed, for example the pH value, may also alter the orientation of the enzyme regarding the support surface [45–47].

Previously, our research group has immobilized ficin extract in glyoxyl activated support, focused only on the stability of lowly loaded enzyme immobilized preparations [40]. In this new research effort, ficin extract has been immobilized on amino and amino glutaraldehyde agarose beads, trying to explore the versatility of glutaraldehyde to get a biocatalyst with high activity and stability. In this new paper, the effect of the protein loading on enzyme activity versus small synthetic substrate (benzoyl-arginine-*p*-nitroanilide (BANA)) and large casein was analysed [1,30,31], as well as the likely effects on enzyme stability, as the loading may affect the distance between different immobilized enzyme molecules and that, in turn, enzyme stability [48,49].

## 2. Results and Discussion

### 2.1. Immobilization of Ficin Extract in MANAE-Agarose

Figure 1 shows the immobilization courses at pH 5, 7 and 9 of ficin on aminated supports. Immobilization was very slow at all studied pH values, with a significant decrease in enzyme activity, although the free enzyme maintained full activity. Yields were higher at pH 9 than at pH 5 or 7, although

expressed activity (the observed activity of the immobilized enzyme compared to the expected one from the immobilization yields data) was always very low (just near to 10%). To check of the yield was determined by the full loading of the support, the supernatant was used in a new immobilization cycle (at pH 7) and a similar immobilization yield was observed (results not shown). This suggest that the yield was not caused by the overloading of the support or by the presence of some enzyme isoforms that cannot be immobilized on the support, suggesting that was due to some kind of adsorption equilibrium. The activity was determined with casein and the small synthetic substrate (BANA), with scarce differences. These facts suggested two contradictory ideas: the ion exchange was so mild that it was unable to fully adsorb the enzyme but the immobilization produces very negative effects on enzyme structure that yielded a decrease versus small and large substrates. Figures 2–5 show the distribution of anionic and main cationic residues on the 4 isoforms of ficin component. In most cases, there is not an area clearly richer in anionic residues than in cationic ones; this may explain why immobilization via ionic exchange is so slow and ineffective. However, in all sides of the proteins there are some Lys groups that could give at least one covalent attachment after ion exchange if treated with glutaraldehyde.

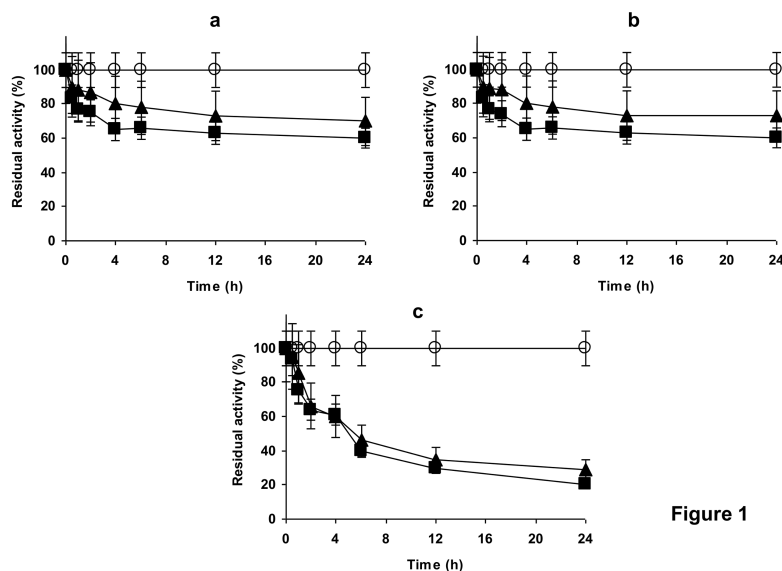
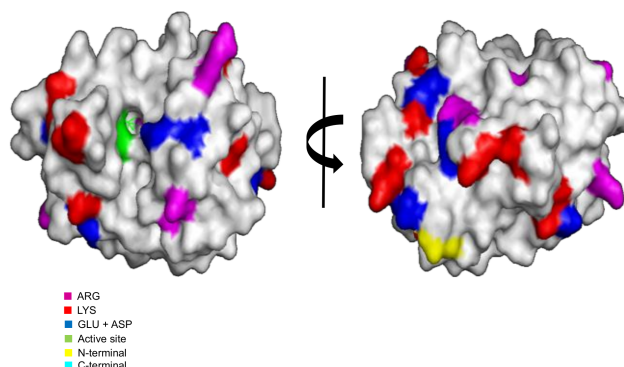


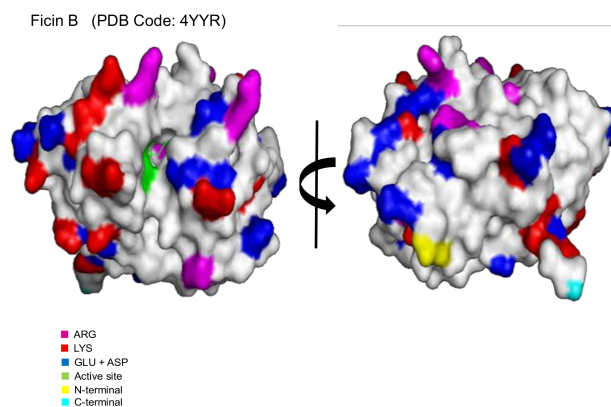
Figure 1

**Figure 1.** Immobilization of ficin extract on MANAE-agarose beads at pH 5 (a), 7 (b) or 9 (c). Immobilization was performed as described in methods. Activity was followed using BANA as substrate. Circles: reference; Triangles: suspension, Squares: supernatant.

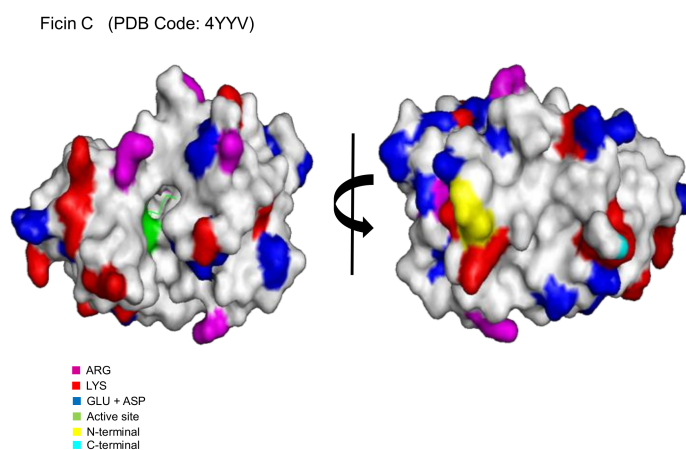
Ficin A (PDB Code: 4YYQ) CHAIN A



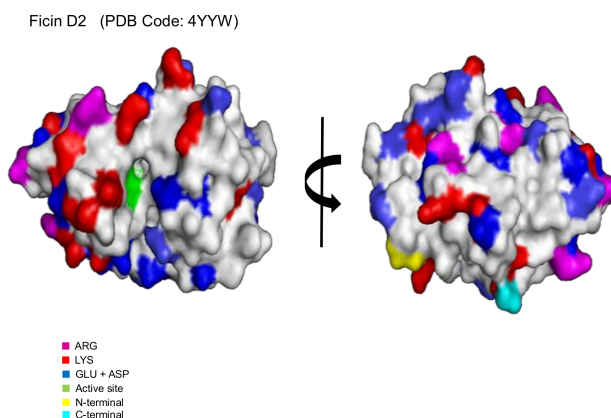
**Figure 2.** 3D surface structure model of Ficin A obtained from the Protein Data Bank (PDB) and displayed using PyMol versus 0.99. Arg, Lys, Glu+Asp, -NH<sub>2</sub> terminal and -COOH terminal residues are indicated.



**Figure 3.** 3D surface structure model of Ficin B obtained from the PDB and displayed using PyMol versus 0.99. Arg, Lys, Glu+Asp, -NH<sub>2</sub> terminal and -COOH terminal residues are indicated.



**Figure 4.** 3D surface structure model of Ficin C obtained from the PDB and displayed using PyMol versus 0.99. Arg, Lys, Glu+Asp, -NH<sub>2</sub> terminal and -COOH terminal residues are indicated.



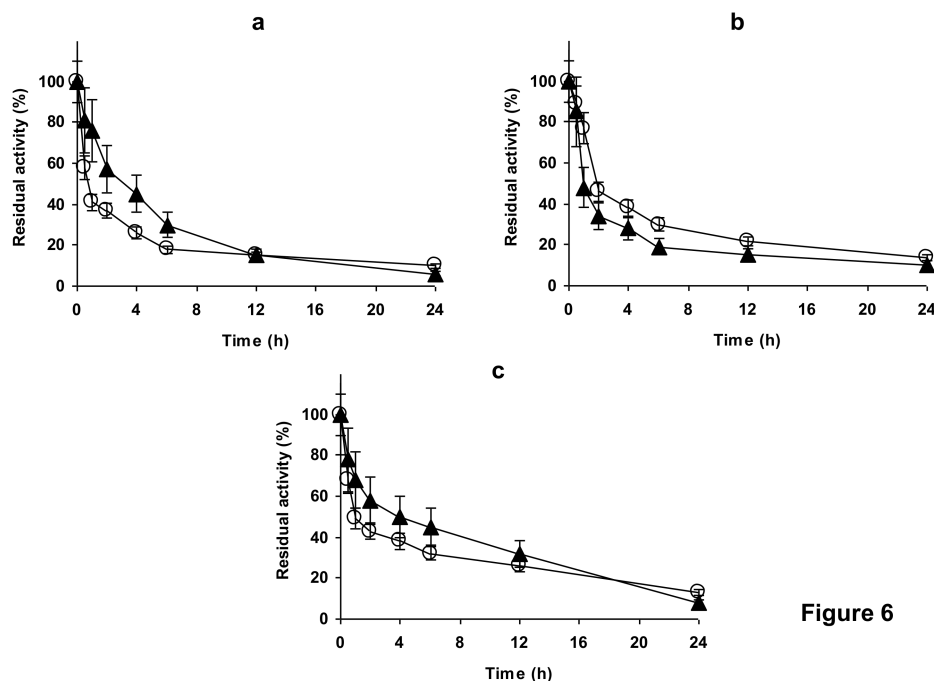
**Figure 5.** 3D surface structure model of Ficin D<sub>2</sub> obtained from the PDB and displayed using PyMol versus 0.99. Arg, Lys, Glu+Asp, -NH<sub>2</sub> terminal and -COOH terminal residues are indicated.

Figure 6 shows the inactivation of the ionically exchanged proteins at pH 7 when inactivated at pH 5, pH 7 and pH 9. A slight stabilization may be found at pH 5 and 9, while a slight destabilization may be found at pH 7. These differences may be based in the way the free enzyme is inactivated. The free enzyme may suffer enzyme autolysis, that way reducing the enzyme stability/activity.



Moreover, the free enzyme may experience protein aggregation (mainly near to the isoelectric point). These two inactivations are not possible using an immobilized enzyme form [30]. In fact, under conditions where aggregation is quite unlikely, stability of the enzyme decreased after immobilization. This may be caused by undesired enzyme-support interactions where inactivation that can stabilize incorrect enzyme structures [29,50,51].

These results were not very positive and anion exchange was discarded as a likely one to immobilize ficin.



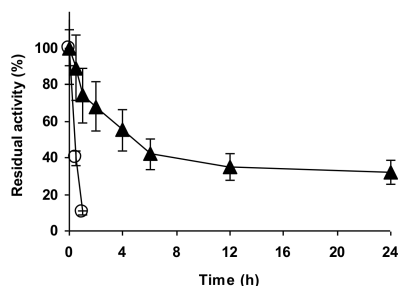
**Figure 6**

**Figure 6.** Inactivation courses of the ionically exchanged ficin extract on MANAE-agarose beads at pH 7. The inactivation studies were performed as indicated in Methods section at pH 5, (a), 7 (b) or 9 (c) at 55 °C, using BANA as substrate. Circles: Free enzyme. Triangles: immobilized enzyme.

## 2.2. Modification of the Ionically Exchanged Enzyme with Glutaraldehyde

In a trial to improve the stability of the ionically exchanged enzyme, the immobilized enzyme was treated with glutaraldehyde, even although the expressed activity of this preparation was already quite low. When the ionically exchanged enzyme was modified with glutaraldehyde, most of the remaining activity was lost, making unsuitable this strategy to improve the enzyme stability.

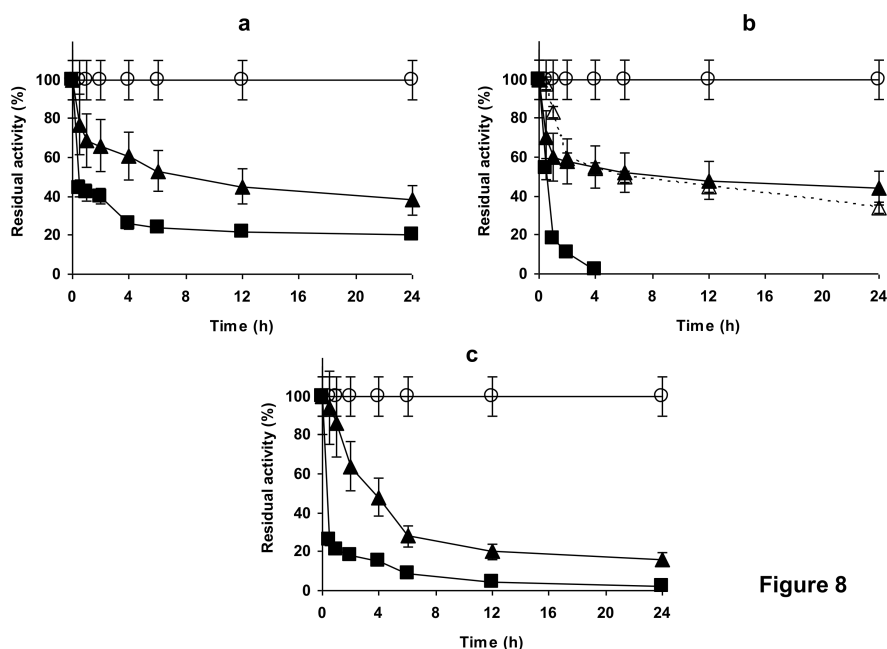
To check if the problem was a consequence of a too intense support-enzyme reaction or the chemical modification of the enzymes with glutaraldehyde, the enzyme immobilized on glyoxyl agarose was modified with glutaraldehyde under the conditions used to modify the adsorbed enzyme. Figure 7 shows how the modification of the glyoxyl-ficin biocatalyst with glutaraldehyde produces some enzyme inactivation, suggesting that the direct modification of the enzyme with this chemical reagent could be partially responsible of the enzyme inactivation. As this preparation was more stable than the free enzyme [40], it may be expected that the enzyme may be also more stable versus chemical modification [52]. However, using the aminated support the residual activity was under 10% after 1 h (Figure 7), suggesting that an inactivation caused by the activated support and enzyme reaction cannot be discarded.



**Figure 7.** Modification of glyoxyl (triangles) or MANAE agarose (circles) immobilized ficin extract when incubated in 1% glutaraldehyde at pH 7. Experiments were performed as described in Methods using BANA as substrate.

### 2.3. Immobilization of the Enzyme in Glutaraldehyde Pre-Activated MANAE Agarose Beads

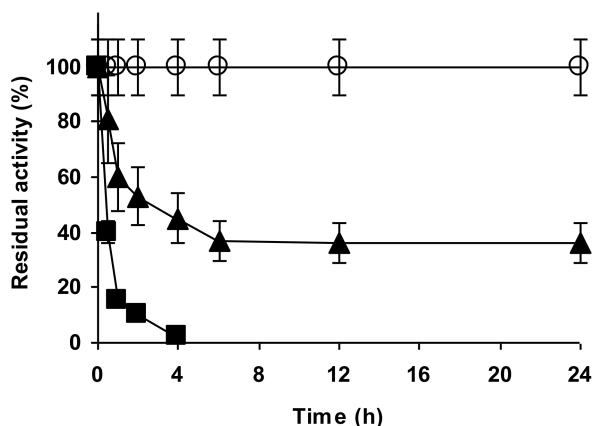
Figure 8 shows the immobilization course at pH 5, 7 and 9 of ficin extract on glutaraldehyde pre-activated agarose beads. At pH 5 immobilization yield was 80% after 24 h and only 20% of the initial activity was present in the immobilized biocatalyst. Thus, this preparation was discarded. At pH 9 the enzyme was almost fully immobilized after 6 h but the enzyme was almost fully inactivated (16% after 24 h). That way, this preparation was also discarded. However, when the enzyme was immobilized at pH 7, full immobilization was obtained after 4 h and the expressed activity was around 40% versus BANA. When the activity was followed versus casein, initially both lines were almost identical, suggesting that immobilized ficin molecules had no reduced accessibility to the casein substrate (that is, the active centre is not oriented towards the support surface). When 24 h of enzyme-support interaction were permitted, the activity versus casein was a 25% lower than versus BANA (about 40% versus about 30%), suggesting that the changes of the enzyme structure during support-enzyme interactions may affect more to the activity versus casein than versus BANA.



**Figure 8**

**Figure 8.** Immobilization of ficin extract on MANAE-agarose beads activated with glutaraldehyde. Experiments were performed as described in the Section 3 using 25 mM of buffer, at pH 5 (a), 7 (b) or 9 (c). Open circles, solid lines: reference; Using BANA as substrate (solid lines, solid symbols): Triangles: suspension, Squares: supernatant. Using casein as substrate (only at pH 7): dotted line, empty triangles.

Many reports stated that covalent glutaraldehyde immobilization is so slow that, if ion exchange was permitted, immobilization proceeded via a first ion exchange, followed by the covalent reaction. Figure 9 shows the immobilization of ficin extract at pH 7 and 200 mM sodium phosphate, where ion exchange was prevented. The immobilization course is pretty similar to the use of 25 mM, suggesting that in both cases the immobilization mainly proceeded via a first covalent attachment. After 4 h, the enzyme could not be desorbed from the support by incubating the biocatalysts in 500 mM NaCl. We analysed the results obtained when immobilizing at pH from 6.5 to 8.5 and the results in terms of activity and stability were optimal at pH 7–8, with a slight advantage at pH 7 (results not shown). Thus, pH 7 was selected as optimal pH for the enzyme immobilization on this support.



**Figure 9.** Immobilization of ficin extract on MANAE-agarose beads activated with glutaraldehyde at pH 7 and 200 mM sodium phosphate using BANA as substrate. Experiments were performed as described in Methods, using BANA as substrate. Circles: reference; Triangles: suspension, Squares: supernatant.

#### 2.4. Stability of the Enzyme Immobilized at pH 7 on Glutaraldehyde Pre-Activated MANAE Agarose Beads

The enzyme immobilized at pH 7 at low and high ionic strength presented a very similar behaviour, thus we have just shown the results obtained with the enzyme immobilized at 25 mM sodium phosphate. Figure 10 shows the inactivation courses at pH 5, 7 and 9, of the free and immobilized enzymes, followed with BANA and casein hydrolyses. Results suggested that a certain stabilization has been achieved using this support but stabilization was clearer at pH 7 than at pH 5 or 9. These differences in stabilization may be due to different interactions of the enzyme and the support—as the support remained as an ion exchanger—or to the fact the enzyme may follow different pathways in the inactivations, that way the immobilization by a specific area may affect enzyme stability in different ways [53]. Using BANA, the immobilized enzyme always seemed to be slightly more stable than when using casein, suggesting that the activity versus the casein is lost before the activity versus BANA. This was more evident at pH 7 than at the other pH values. The situation was not so evident using the free enzyme, where inactivations were very similar with both substrates at pH 5, while at pH 7 and 9 higher stability using casein as substrate was observed. In fact, stabilization at pH 5 and 7 caused by the enzyme immobilization was smaller using casein as substrate than using BANA. This finding may be related to different changes on the enzyme structure that may affect more significantly to the hydrolysis of one substrate or other one.

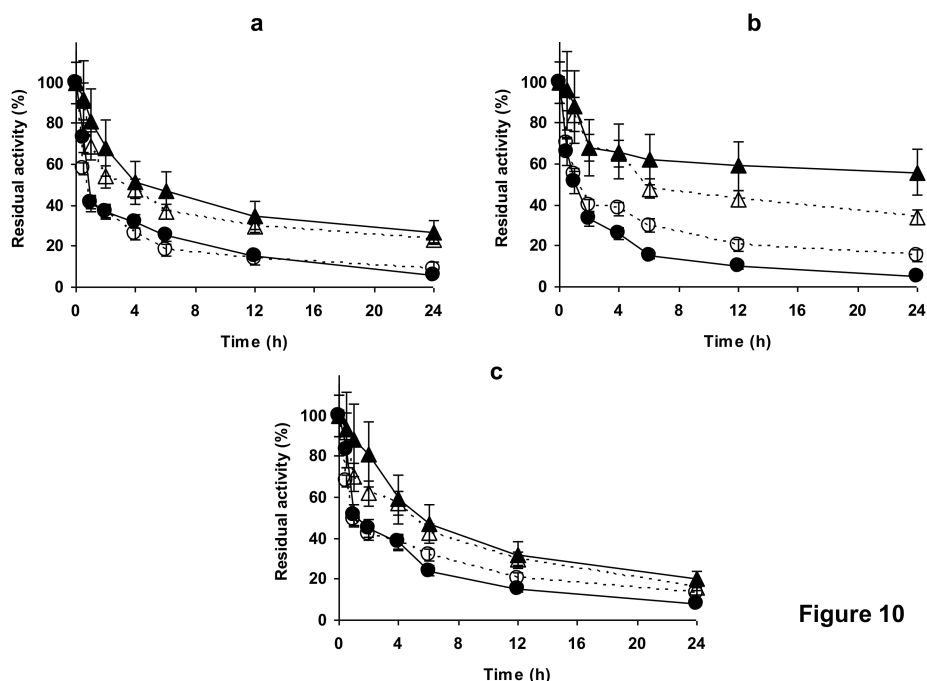
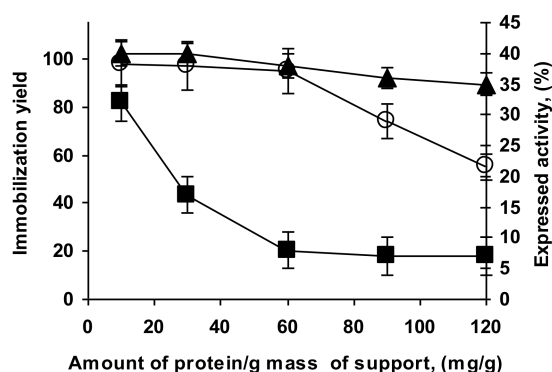


Figure 10

**Figure 10.** Inactivation of ficin immobilized at pH 7 using 25 mM sodium phosphate. Experiments were performed as described in Section 3, at pH 5 (a), 7 (b) or 9 (c) and 55 °C. Solid lines and solid circles: inactivation was followed using BANA, Dotted line and empty symbols: inactivation was followed with casein. Circles: free enzyme; Triangles: immobilized enzyme.

### 2.5. Determination of Loading Capacity

Figure 11 shows the immobilization yields when the amount of enzyme was increased using BANA and casein. From these experiments, maximum loading was established in the range 68–72 mg ficin/g of support. Expressed activity per mg of enzyme versus the small synthetic substrate was slightly lower when using higher enzyme loadings, although the values were pretty similar. The small differences may be attributed to an increment of the diffusion limitations.



**Figure 11.** Immobilization yield and expressed activity when growing the amount of offered ficin extract per g of MANAE activate with glutaraldehyde support. Experiments were carried out as described in Methods section. Triangles: immobilization yield; Circles: expressed activity versus BANA, Squares: expressed activity versus casein.

One critical point using proteases to hydrolyse proteins, as stated in the introduction, is that the loading of the support with the enzyme may generate some steric hindrances that prevent the accessibility of the large substrate to the enzyme if the orientation is not perfect towards the reaction medium [1]. Figure 11 shows that using casein the specific activity of the immobilized ficin

extract rapidly decreased with increasing loading, going from slightly more than 30% of the activity immobilized at 10 mg/g to around 15% using 30 mg/g and only 7–8% using maximum loading or overloading. In fact, the observed activity per gram of biocatalyst was maximal using 30 mg/g, using higher enzyme loading the biocatalyst mass activity decreased by 30–35%. This suggested that the enzyme active centre was not perfectly looking toward the opposite side of the support surface and that way the nearby enzyme molecules may cause steric hindrances to the entry of the large substrate casein when the support was fully loaded. However, this limitation only affected the use of the biocatalyst in the hydrolysis of proteins, not in the other applications that the enzyme may have and that involve small substrates (see Section 1).

Another point that may have interest was to determine if the loading may somehow affect the immobilized ficin enzyme molecule stability. If the immobilization rate is high enough, it is likely that some molecules may be packed together and near enough to interact with each other, altering the final stability properties in a positive or a negative way, depending on the enzyme and inactivation conditions [48,49]. However, when we analysed this effect, we did not find any significant difference among the different preparations, just a very slight higher stability of the more loaded preparations; differences are scarce and may be attributed to diffusional limitations (e.g., after 6 h, the biocatalyst having 1 mg/g retained a  $55 \pm 2\%$  of the initial activity, while the one prepared using 12 mg retained  $60 \pm 2\%$ ).

Using the preparation with 1 mg/g in hydrolysis of casein, we have reused the biocatalyst in 6 consecutive cycles of casein hydrolysis at 50 °C and pH 7 for 2 h without detecting any significant change in the biocatalyst performance (not shown results).

### 3. Materials and Methods

#### 3.1. Materials

Glycidol, 25% (v/v) glutaraldehyde solution, sodium borohydride, sodium periodate, ethylenediamine, benzoyl-arginine-p-nitroanilide (BANA), cysteine, bovine serum albumin (BSA) and casein were purchased from Sigma-Aldrich (St. Louis, MO, USA). Agarose beads 4 BCL support was purchased from Agarose Bead Technologies (ABT), Madrid, Spain. All other reagents were of analytical grade. All experiments were performed by triplicate and the reported values the mean of the results of this set of experiments with their standard deviation. Glyoxyl agarose beads were prepared as previously described [54,55]. MANAE- supports was prepared from glyoxyl supports with a modification of the protocol previously described [56,57], ethylenediamine/glyoxyl agarose beads reaction time was 24 h before reduction. Glyoxyl-ficin was prepared using 1 mg ficin/g of support as previously described [40].

#### 3.2. Preparation of Glutaraldehyde Agarose Beads

50 grams of MANAE agarose beads was suspended in 100 mL of 15% (v/v) glutaraldehyde in 200 mM phosphate buffer pH 7.0. The suspension was gently stirred 14–16 h at 4 °C. After that, the activated support was washed with distilled water. The activated support was used immediately after preparation. This protocol guarantees that each primary amino in the support has been modified with two glutaraldehyde molecules [44,58,59].

#### 3.3. Preparation of Ficin Extract

Fresh fig latex was obtained breaking fresh immature green fruits and leaves of *Ficus carica* L. The samples were picked in Kabylia, north of Algeria (Adekar, Bejaia). The fluid was collected in a clean flask at 4 °C. The latex was centrifuged at  $3200 \times g$  for 15 min at 4 °C [60] to eliminate debris (e.g., gums). The supernatant was used as “crude extract of ficin” (with a concentration of 98.5 mg protein/mL). The extract contains the four protease isoforms and it is similar to the usually utilized in ficin applications. A SDS-PAGE may be observed in support ting information. It was stored at  $-20\text{ }^{\circ}\text{C}$



until use. The protein concentration was quantified by Bradford's method [61]. BSA was used as the standard protein. The activity of the free enzyme versus casein (see below) was  $5 \pm 0.7$  units/mg of protein.

### 3.4. Enzymatic Assays

Activity of immobilized and free ficin extract was determined using casein and benzoyl-D, L-arginine *p*-nitroanilide hydrochloride (BANA) as enzyme substrates.

The enzyme activity determination using BANA was performed as previously described [29], BANA solution was prepared by dissolving 43.5 mg of BANA in 1.0 mL of dimethyl sulfoxide and diluting to 100 mL in 0.1 M sodium phosphate pH 7, containing 5 mM EDTA. The enzyme activity was assessed by measuring the *p*-nitroaniline released at 405 nm (under these conditions, the  $\epsilon$  for *p*-nitroaniline was 8800). Activity of ficin was expressed as  $\mu$ mol of *p*-nitroaniline released per mg of extract and min.

The enzyme activity using casein was determined as described by Kunitz, with some modifications [62]. A solution of 1% (*w/v*) casein was prepared in 100 mM sodium phosphate at pH 7.0 containing 5 mM cysteine hydrochloride and 5 mM EDTA at 55 °C. To 1 mL of this substrate solution, 100  $\mu$ L of ficin (enzyme extract solution or immobilized ficin suspension) was added and the reaction mixture was incubated at the desired temperature for 20 min. The reaction was stopped by the addition of 1 mL of 10% trichloroacetic acid (TCA), incubated for 10 min at room temperature and centrifuged at 10,000 rpm. This treatment produces the precipitation of the remaining protein but the peptides remained in solution. The absorbance of soluble peptides in the supernatant was measured at 280 nm. In the case of the reference, substrate was added after the enzyme was first inactivated by incubation in TCA. One unit of activity is defined as increment in absorbance of 0.001 per mg of ficin and min under the given assay conditions.

### 3.5. Immobilization of Ficin Extract

Ten grams (10 g) of the corresponding support (MANAE-agarose or glutaraldehyde-agarose) were added to 100 mL of ficin extract (containing 1 mg protein/mL) in 25 mM sodium acetate at pH 5, 25 mM sodium phosphate at pH 6.5–8.5 or 25 mM sodium carbonate at pH 9, in all cases the temperature was 25 °C. In some instances, the enzyme concentration was increased to increase the amount of offered enzyme (a maximum of 12 mg/mL was employed). Samples from suspensions and supernatants were periodically withdrawn and their catalytic activity determined using BANA and casein. After 24 h, the biocatalysts were vacuum filtered and washed thoroughly with distilled water.

### 3.6. Enzyme Inactivation Studies

1 g of immobilized enzyme was suspended in 10 mL of 50 mM in the corresponding buffer at 55 °C (sodium acetate at pH 5, sodium phosphate at pH 7 or 50 mM sodium carbonate at pH 9). For the free enzyme extract, 1 mg/mL of ficin solution was prepared in the same buffer and temperature. Samples were periodically withdrawn and the activity determined using the BANA and casein assay described above.

### 3.7. Reuse of the Immobilized Ficin in the Hydrolysis of Casein

Six cycles of casein hydrolysis (2 h each) were performed at 50 °C and pH 7. After 2 h of casein proteolysis, the peptide production was checked as described above and the immobilized enzyme was washed 5 times with 10 volumes of distilled water and employed in a new reaction cycle.

## 4. Conclusions

Ficin extract may be immobilized on glutaraldehyde activated supports, best results in terms of immobilization yield and expressed activity are achieved when the enzyme is immobilized at pH 7.

Immobilization at 25 or 200 mM sodium phosphate is relatively similar, while the aminated support is unable to immobilize more than 30–40% of the enzyme. This suggested that the first immobilization step is in both cases the covalent attachment of the enzyme. Immobilization at pH 5 failed in permitting full enzyme immobilization, while at pH 9 the enzyme become inactivated. Stabilization depended on the pH and the substrate used to determine the residual activity, being larger when using BANA and shorter using casein. Results suggest a complex net of interactions between enzyme and support that differently affect the activity versus the different substrates. For the hydrolysis of casein, enzyme specific activity drops rapidly using high loadings, while it is almost identical using BANA. Enzyme loading has not a significant effect on immobilized enzyme stability.

**Supplementary Materials:** The following are available online at <http://www.mdpi.com/2073-4344/8/4/149/s1>, Figure S1: SDS PAGE of ficin extract (1 mg/ml). Lane 1 Molecular weight markers, Lane 2, 3, 4 different ficin extracts used in this paper.

**Acknowledgments:** We gratefully recognize the support from the MINECO from Spanish Government, (project number CTQ2017-86170-R). El-Hocine Siar thanks the Algerian Ministry of Higher Education and Scientific Research for his fellowship. The help and suggestions of Ángel Berenguer (Instituto de Materiales, Universidad de Alicante) are gratefully recognized.

**Author Contributions:** R.F.-L. and M.N.Z. conceived the experiments; R.F.-L. designed and supervised the experiments, E.-H.A., S.A.-P. and O.B. performed the experiments, E.-H.A., S.A.-P. and R.F.-L. wrote the paper.

**Conflicts of Interest:** The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript and in the decision to publish the results.

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