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Changes in ficin specificity by different substrate proteins promoted by enzyme immobilization

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ABSTRACT

Ficin extract has been immobilized using different supports: glyoxyl and Aspartic/1,6 hexamethylenediamine (Asp/HA) agarose beads. The latter was later submitted to glutaraldehyde modification to get covalent immobilization. The activities of these 3 kinds of biocatalysts were compared utilizing 4 different substrates, casein, hemoglobin and bovine serum albumin and benzoyl-arginine-p-nitroanilide at pH 7 and 5. Using glyoxyl-agarose, the effect of enzyme-support reaction time on the activity versus the four substrates at both pH values was studied. Reaction time has been shown to distort the enzyme due to an increase in the number of covalent support-enzyme bonds. Surprisingly, for all the substrates and conditions the prolongation of the enzyme-support reaction did not imply a decrease in enzyme activity. Using the Asp/HA supports (with different amount of HA) differences in the effect on enzyme activity versus the different substrates are much more significant, while with some substrates the immobilization produced a decrease in enzyme activity, with in other cases the activity increased. These different effects are even increased after glutaraldehyde treatment. That way, the conformational changes induced by the biocatalyst immobilization or the chemical modification fully altered the enzyme protein specificity. This may also have some implications when following enzyme inactivation.

1. Introduction

Proteases may be utilized for many different purposes, being one of them the hydrolysis of proteins, to produce bioactive peptides, free amino acids, eliminate allergenic proteins, etc. [1–10] Each protease bears different specificity and selectivity [10–19]. That way, there are carboxy or amino exo-proteases and endo-proteases. Proteases may also be specific for precise sequences or amino acids in the amino or carboxy position of the protein chain [10–19]. In many instances, this can determine their successful application. One protease can hydrolyze many different proteins, but usually at different rates as a function of the protein sequence, exposition of specific groups, flexibility of the protein structure, etc., giving different peptides that can produce hydrolysates with different bioactivities [5,20–24]. That way, it may be expected that a specific protease can hydrolyze more rapidly one specific protein than another, depending on the enzyme and substrate protein features. Furthermore, we can consider that it may be interesting to find strategies to alter this protein substrate specificity for some specific applications.

We should also consider that the use of immobilized enzymes presents some advantages compared to the use of free ones [25–27]. For example, it simplifies enzyme recovery, avoiding their becoming part of the final product. The use of immobilized enzyme not only enables a strict control of the reaction, but also prevents some negative effects on the consumer by the addition of the utilized proteases (e.g., allergenic effects) [28–31]. Moreover, free protease molecules may attack other free enzyme molecules, promoting their inactivation and also incorporating the produced peptides and amino acids to the final product. Using proteases immobilized on porous supports, this autolysis is almost fully avoided (only the extremely small fraction of enzyme molecules on the surface of the beads can suffer proteolysis) [32–36]. Moreover, a proper

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immobilization protocol may produce an increase of the enzyme structure rigidity and, that way, an increase in the enzyme stability in the presence of distorting agents [34,37–48]. This stabilization permits to increase the number of biocatalyst reuses in operation, and also increases the range of conditions where the enzyme can be utilized [49] (e.g., if the use of caotropic agents is required to redissolve protein aggregates and permit its hydrolysis)[50,51]. Enzyme rigidification may be achieved by multipoint covalent immobilization, as this fixes the relative positions of all the groups involved in the immobilization [49]. To get optimal enzyme-support multipoint covalent attachment requires utilizing a suitable immobilization protocol [49]; a random immobilization protocol can lead to get an even less stable enzyme. Enzyme stabilization by enzyme immobilization may be also caused by generation of favorable enzyme nano-environments [42,52–57], multisubunit immobilization (in multimeric enzymes)[58], fixation of more stable structures (e.g., the open and adsorbed open form of lipases)[59,60], etc. Immobilization can be also coupled to enzyme purification if the protocol is properly designed [61–70]. The immobilization of the proteases inside porous supports can generate some substrate diffusional limitations [71–74], and the presence of the support surface near the enzyme can originate some steric hindrances towards the contact between the active center of the enzyme and the protein substrate [32–36]. Both phenomena may alter the enzyme performance.

Recently, it has been reported that the promotion of tailor-made steric hindrances can alter the protein substrate size-specificity of the immobilized proteases, greatly decreasing the activity versus large proteins while scarcely affecting the activity versus proteins of a size similar to that of the immobilized protease molecule [75,76] Furthermore, the interactions between the enzyme and the support (those that cause the enzyme immobilization or those that are established after its immobilization if the support surface is not fully inert, by the proximity of the enzyme and the support surface), can distort the enzyme structure. These changes are almost inevitable [77] and have enabled to alter the specificity and selectivity of many enzymes via immobilization [34,35,78–90]. However, we have not been able to find examples of tuning protease specificity by different proteins via immobilization, usually attributed the exposition of target sequences to the action of the protease. To study if also the changes on enzyme conformation can affect the recognition of the substrate protein by immobilized protease (as it is not easy to think in changes on sequence specificity) is the main topic of the current research. As model protease, we have utilized ficin, a protease extract from *Ficus carica* [91,92]. This is a thiol protease that has many applications in diverse areas (tenderization of meat [93–95], production of cheese [96,97], production of bioactive peptides [98–102], etc.). The active components ratio are altered by many factors, including the ambient and watering, the watering, the health of the tree, the land physicochemical properties, etc.[103,104]. For instance, during fruit ripening the protein content increase, but the content of ficin decreased. [105]. The enzyme has been immobilized on glyoxyl-agarose[50,106,107] (Fig. 1), detecting that if the enzyme-support reaction was permitted to progress for a long time, enzyme stability decreased since the catalytic Cys became more exposed and was oxidized by oxygen [108]. This result suggested conformation changes in the proximity of the active center of the immobilized enzyme, and to check possible changes in the ficin protein-specificity caused by these changes may be interesting. Moreover, recently ficin has been immobilized on different mixed Asp/1,6-hexamethylenediamine supports via the enzyme ionic exchange followed by glutaraldehyde treatment to crosslink the enzyme and the support (Fig. 1), enabling the enzyme covalent immobilization and stabilization[109]. To get the enzyme-support immobilization, it has been shown that the best conditions are whose where each amino group in the enzyme and the support are modified by one molecule of glutaraldehyde, while this group hardly react with fee amino groups, readily react with other amino-glutaraldehyde group [110–112]. Prof. Monsan reported a long time ago how was possible to get this modification[113]. The immobilized ficin extract is able to produce mainly free amino acids and dipeptides in the hydrolysis of casein for 24 h (unpublished results), showing the low sequence specificity of this proteolytic extract. We have utilized these biocatalysts in this new research effort.

As substrate proteins, we have utilized three different ones with different size or structure, such as casein (with a size of 23 kDa) [114–116], bovine serum albumin (BSA) (with a size of 60 kDa, no globular protein) [117–119] and hemoglobin (HG) (tetrameric protein with a size of 64 kDa and a globular shape)[120,121].

2. Materials and methods

2.1. Materials

Agarose Bead Technologies (Madrid, Spain) provided the 4 BCL agarose beads. Trichloroacetic acid solution (TCA), L-aspartic acid, dimethyl sulfoxide (DMSO), glycidol, glutaraldehyde solution (25 % v/v), 1,6 hexamethylenediamine (98 % v/v) (HA), benzoyl-arginine-p-

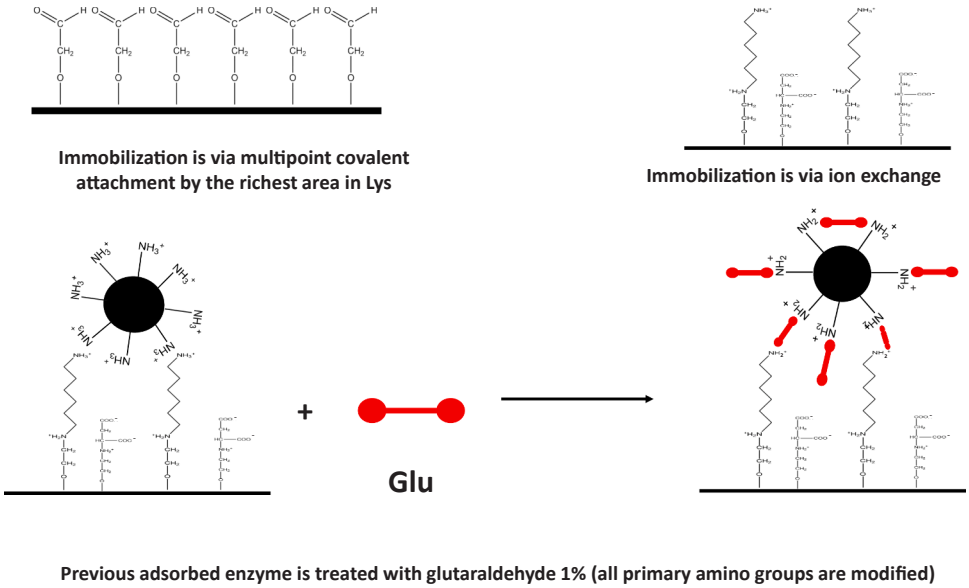

Fig. 1. Schematic representation of the support utilized in this paper.

nitroanilide (BAPNA), hemoglobin (Hg), casein (CS) and bovine serum albumin (BSA) were acquired from Sigma-Aldrich (St. Louis, MO, USA). For all experiments, we employed analytical grade reagents.

2.2. Methods

All experiments in this work were performed in triplicate; the reported results are the mean of these values, including their respective standard deviation.

2.2.1. Preparation of the different supports

The protocol reported by Mateo et. al. and Grazu et. al. [121,122] was employed to obtain glyoxyl agarose beads. These beads were incubated in a 2 M solution of L-Aspartic [123] acid in a 1:10 ratio with different concentrations of HA solution (0, 25 mM, 50 mM, 100 mM, 500 mM, 1 M and 2 M) or in a solution containing the same different concentration of HA mentioned before at pH 10.05 for 24 h at 25ºC under mild shaking. Finally, a mass of 10 mg/mL of solid sodium borohydride was added. After 30 minutes, the modified supports were vacuum filtered and rinsed with an excess of distilled water.

2.2.2. Ficin immobilization via ion exchange on the different supports

A mass of 5 g of support (Asp, HA or Asp/HA) was suspended in 50 mL of 1 mg/mL of ficin solution prepared in 5 mM sodium phosphate buffer solution at pH 7 and 25°C. Then the mixture was stirred in a roller for 24 h at 25ºC. Samples of suspension and supernatant were taken at different times to analyze their activities to determine immobilization yields and expressed activities [122]. An enzyme suspension using inert agarose under identical conditions was used as reference (no adsorption of the enzyme on this support was observed).

2.2.3. Modification of immobilized ficin with glutaraldehyde

Samples of 2.5 g of the immobilized enzyme were incubated in 25 mL of 1 % (v/v) glutaraldehyde solution prepared in 50 mM sodium phosphate at pH 8.0 and submitted to gentle stirring for 1 h at 25ºC. Then, the glutaraldehyde modified samples were filtered and washed with 5 mM sodium phosphate at pH 8.0. The modified biocatalysts were resuspended in 5 mM sodium phosphate buffer solution pH 8.0 at 25°C for 24 h to permit the covalent reaction between amino-glutaraldehyde groups. After that, the biocatalysts were filtered, rinsed with distilled water and stored at 6°C until its use.

2.2.4. Enzyme activity assays

97 mg of BAPNA was dissolved in 2 mL of DMSO. Then, 198 mL of 0.1 M sodium phosphate at pH 7 (standard assay) or sodium acetate at pH 5 containing 5 mM of EDTA and 5 mM of cysteine were added. The hydrolysis of BAPNA was studied following the protocol described by Siar et. al. [51]. The augmentation of absorbance at 405 nm and 55°C caused by the release of p-nitroaniline utilizing an UV–vis spectrophotometer was quantified.

The proteolytic activity of ficin using BSA, CS and Hg was carried out utilizing the method reported by Kunitz [124] (with minor alterations). A volume of 1 mL of substrate at 37ºC and pH 7 or 5 was prepared (1 % of protein prepared in 50 mM phosphate pH 7 or 50 mM sodium acetate pH 5 buffer containing 5 mM of EDTA and 5 mM of cysteine). Then, we added 100 μL of immobilized biocatalyst suspension or free ficin solution and the reactions were left to proceed for 15 minutes. The reaction was stopped by adding 2 mL of 10 % TCA solution. This also promoted the proteins to precipitate, except small peptides that were generated by proteolysis. This was submitted to centrifugation at 10000 g for 15 min at 4°C and the supernatant absorbance was quantified at 280 nm. Proteolytic activity ratio of the different biocatalysts is defined as the activity with the studied protein divided by the activity versus casein.

3. Results and discussion

3.1. Preparation of the biocatalysts

Fig. 2 shows the immobilization courses of ficin at 10 mg of ficin extract/g of support. Immobilization is quite rapid and the activity versus BAPNA slowly decreased along time. Fig. 3 shows the immobilization courses in the different Asp and ASP/HA supports, also at the same enzyme loading. The immobilization rate decreased when the amount of HA in the support increased, as it made the cation exchange of the ficin on the support more difficult [109]. Activity recovery versus BAPNA was similar in all cases.

3.2. Specificity of free ficin by different protein substrates at different pH values

Table 1 shows the effect of the pH and substrate nature on the activity of free ficin. Using the synthetic substrate BAPNA, the change of pH 7 to pH 5 produced a decrease in the enzyme activity by a 30 %. This suggested that the enzyme active center may be more suitable to hydrolyze anilines at pH 7 than 5.

At pH 7 (Table 1), free ficin exhibited the highest activity using CS, using HG the activity decreased to 70 %, while using BSA the activity decreased to 40 %. The situation is different at pH 5, the best substrate became HG (the enzyme activity slightly increased at pH 5 compared to the value at 7 (by less than 5 %)), being the activities with BSA and CS similar (less than 30 % of the activity detected using HG) and lower than at pH 7 (to around 50 % for BSA and 22 % using CS). These drastic changes of the protein specificity of the free ficin just by lowering the pH from 7 to 5 may be consequence of the status of the substrate protein, perhaps some aggregation may be produced for BSA or CS [123–125] While HG may be present in a more dissociated form, also some conformational changes in the substrate protein can produce a higher exposition of the protein to the protease. The effect of pH on the free ficin could also be related to the isoelectric point of the enzyme and the substrate protein. In fact, the results can agree with this possibility. The enzyme has a very high isoelectric point (over 10), that way will be a cationic polymer at pH 7 and 5. BSA and CS will be in anionic form at pH 7, while at pH 5 they will have also the same amount of cations and anions. This way, the adsorption of the substrate protein on the enzyme could be one of the reasons to have more influence of pH for Cs and BSA than for HG. However, this should mainly affect the affinity of the enzyme for the substrate, and will be relevant if the adsorption is produced in the areas around the active center of the enzyme and offering the bonds that can be hydrolyzed by ficin. Moreover, 50 mM of buffer can make complex the adsorption of the substrate protein on the enzyme. The conformational changes of the protease enzyme caused by the change in the medium may also have some influence on the activity versus the different proteins. In any case, these results shows that the optimal conditions for the hydrolysis of a protein depends on the substrate protein, here pH 5 and pH 7 provide similar activities using HG but not using BSA or CS.

3.3. Effect of the immobilization time on glyoxyl agarose beads on the activity of ficin with different protein substrates at different pH values

Next, we compared the activities of different glyoxyl biocatalysts versus different substrates at pH 5 and 7. Table 2 shows that the enzyme-support reaction time decreased the activity versus BAPNA at pH 7 (from 64 % using the biocatalysts of 1 h of immobilization to 52 % after 24 h at pH 7). Using CS as substrate at pH 7, the activity also decreased with incubation time; after 1 h of immobilization, over 47 % of the activity of the free enzyme is observed, and this decreased to 40 % after 24 h. These values are similar to those found using BAPNA. However, the effect of the incubation time on the activity using BSA or HG presented a different profile. Surprisingly, using HG as substrate, after a decrease when the

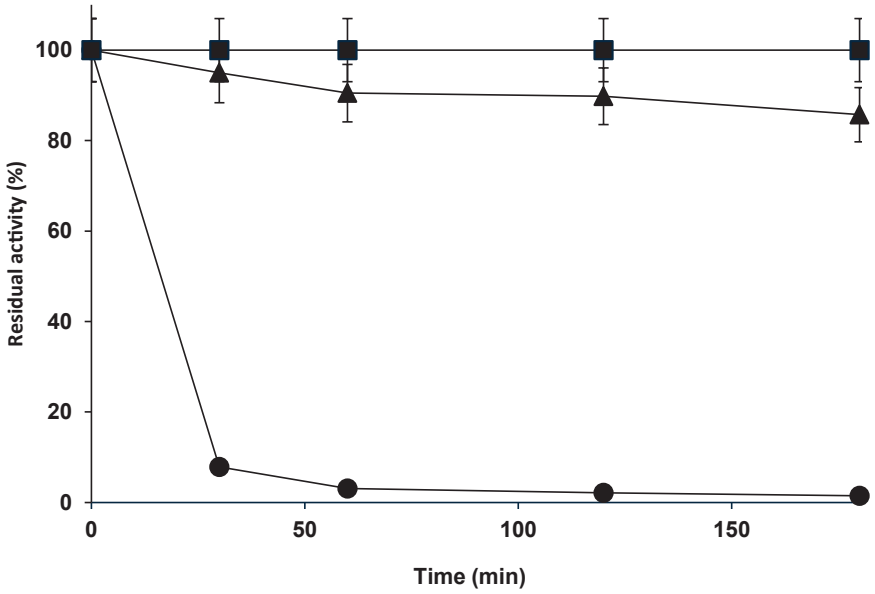

Fig. 2. Immobilization course of ficin on glyoxyl-agarose (10 mg ficin/g support). Immobilization was performed at pH 10 and room temperature. Square: reference; Triangle: immobilization suspension, Circle: supernatant of the immobilization suspension, Other specification are included in Methods section.

enzyme is immobilized, the activity slightly increased along with immobilization time; after 1 h the activity compared to that of the free enzyme increased by 50 % and this figure increased further to 62 % after 24 h. The incubation time almost had no effect using BSA (the activity remains around 70 % in all cases). That, way, it seems that the distortion of the enzyme caused by the immobilization has not the same effect on the activity of the immobilized ficin extract. In this instance, the only explanation should be based on the conformational changes of the enzyme during the multipoint covalent reaction, as any alteration in the substrate protein must be constant, and the ionization of the immobilized ficin is almost the same (just changing a primary amino group by a secondary amino group).

At pH 5 (Table 3), the activity progressively decreased versus BAPNA when the incubation time was prolonged, and the activity was lower than at pH 7, similarly to the results observed using the free enzyme. However, if the activity is determined using CS, the immobilization produced a positive effect on the activity of ficin, almost showing 140 % of that of the free enzyme at pH 5 (however, it still remained less active than at pH 7), and almost remained unaltered during the multipoint covalent immobilization. Similar effects of the immobilization and the incubation time were observed using BSA as substrate, in this instance the increase in activity was 26 %. Very interestingly, the immobilized ficin presented a similar activity using this substrate at pH 7 and 5, while the free enzyme decreased the activity by 50 %. The situation was different using HG as substrate, the biocatalysts prepared for 1 and 3 h presented around 45 % of the activity of the free enzyme, but the biocatalyst prepared by reaction during 24 h, increased its activity to more than 65 % (Table 3). Again very different effects of the incubation time could be found using different substrates to determine the enzyme activity, and the only explanation is that the conformational changes of the enzyme during the multipoint covalent attachment affected the action of the immobilized ficin versus different substrates in a different form. Altogether, the results suggested that a great contribution of the effect of the pH on free ficin activity versus different proteins may be caused by the change of the ficin structure promoted by the pH value. The immobilized/stabilized ficin may be more resistant to these changes.

As result of these, the immobilization of ficin immobilized on glyoxyl agarose beads presented different ratios on the hydrolysis rate of the different proteins. The free enzyme at pH 7 presented activity ratios (regarding the activity versus casein) of 0.7 with HG and 0.3 with BSA. The enzyme immobilized for 1 h gave values of 0.77 for HG and 0.6 for BSA; after 3 h, these values were 0.94 for HG and 0.63 for BSA, and after 24 h, 1.1 for HG and 0.74 for BSA. That way, the activity ratio of the different biocatalyst was different for each biocatalyst, and these differences increased with incubation (that is, distortion of the enzyme) time.

At pH 5, the effects of the immobilization were also very clear. The value of the free enzyme for this activity ratio was 3.3 for HG and 0.9 for BSA (very different to that found at pH 7. The enzyme immobilized for 1 h or 3 h gave a value of around 1 for HG and 0.9. the biocatalyst prepared for 24 gave a value of 1.6 for HG and 0.8 for BSA.

3.4. Effect of the support HA/Asp ratio and the treatment with glutaraldehyde on the activity of ficin with different protein substrates at different pH values

The activity of ficin immobilized on the different Asp/HA supports has been also evaluated. The effect of the glutaraldehyde treatment has been also included, as this can promote some conformational changes by the covalent bonds or by the chemical modification.

First, we will analyze the activity of the lowly loaded and just ionically exchanged ficin biocatalyst versus the different substrates at pH 7 (Table 4). Regarding the use of BAPNA as substrate, the activity decreased from almost 1.4 to just over 0.5 after immobilization on Asp supports, all Asp/HA gave similar values, around 0.31. Passing to the proteolytic activity, we should consider the possibility of the support adsorbing the substrate protein (although this should be increased using a support with one amino groups). If this adsorption is produced, the adsorbed protein will be not available for the enzyme and this could somehow decrease the detected enzyme activity (although we are using a very high substrate protein concentration). To analyze this possibility, the adsorption of the substrate protein on the used supports was analyzed, and we did not found any relevant adsorption on the support. The enzyme adsorbed on agarose beads bearing only Asp groups presented similar activity with HG and CS (activity was lower than that determined using the free enzyme), with around 55 % of activity versus BSA (the activity versus this protein was higher than that of the free enzyme (Table 1)). The use of Asp/HA groups in the support decreased the activity versus HG, while it significantly increased the activity versus CS. However, when the amount of HA groups increased in the support, the activity versus CS slightly decreased, while when using HG as substrate, the only a relevant change was found with the enzyme

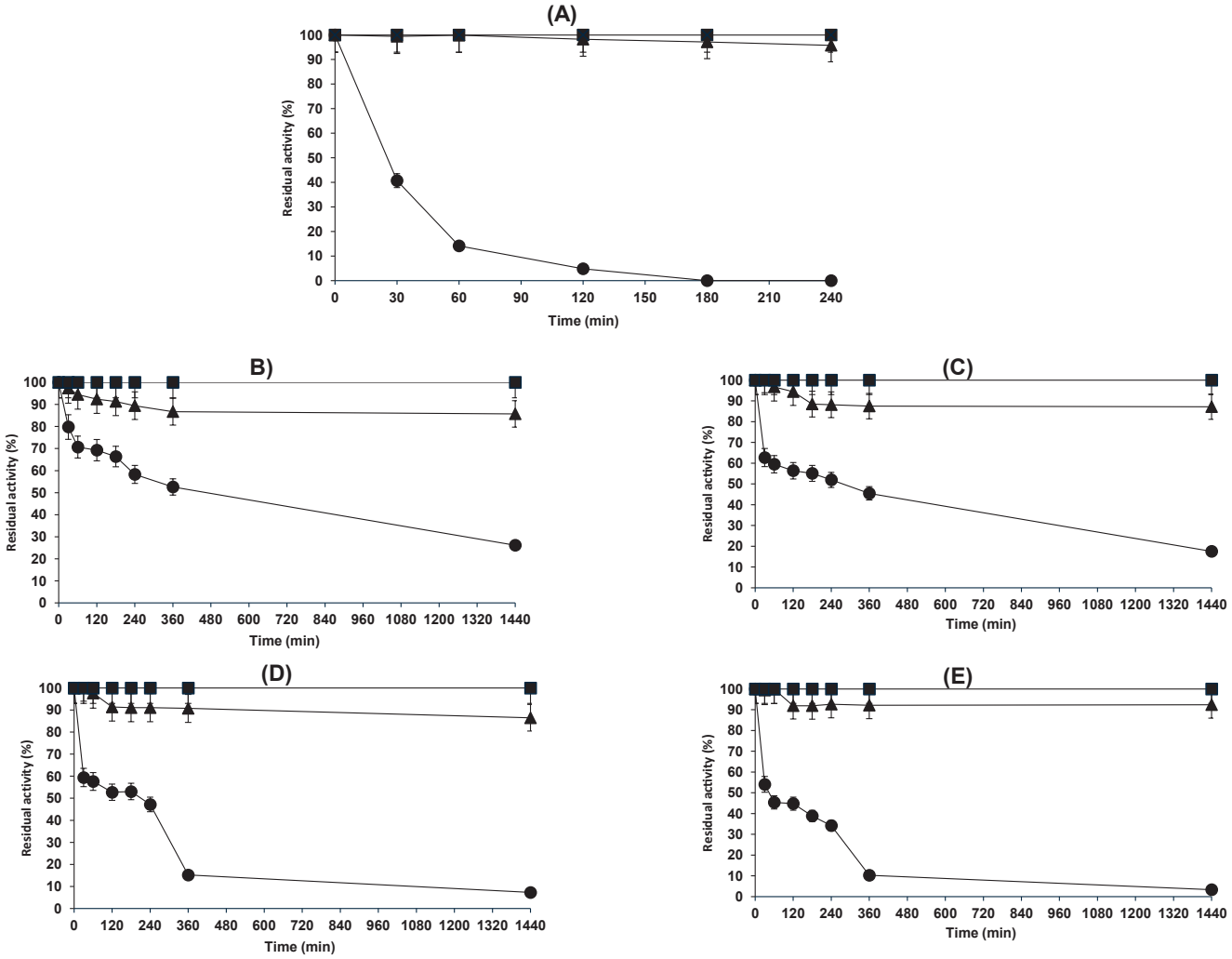

Fig. 3. : Immobilization course of ficin in **(A)** Aspartic-agarose, **(B)** 2 M Aspartic + 0,5 M Hexamethylenediamine Agarose, **(C)** 2 M Aspartic + 0,1 M Hexamethylenediamine Agarose, **(D)** 2 M Aspartic + 0,05 M Hexamethylenediamine Agarose and **(E)** 2 M Aspartic + 0025 M Hexamethylenediamine Agarose, Immobilization was performed as described in Materials and methods section. Squares: reference; triangles: immobilization suspension; solid circles: supernatant of the immobilization suspension.

Table 1
Activity of free ficin using different substrates at pH 7.0 and 5.0 and 37°C. In the protease activity, the absorbance obtained under the conditions detailed in the Methods section is used. Activity is given as increment of Absorbance at 280 nm after 15 minutes of reaction (see methods section).

Substrate	CS	HG	BSA	BAPNA
pH 7	0.94 ± 0.05	0.67 ± 0.02	0.38 ± 0.01	1.38 ± 0.02
pH 5	0.21 ± 0.03	0.69 ± 0.02	0.19 ±0.01	0.96 ± 0.02

Table 2
Activity of different glyoxyl-ficin biocatalysts (1, 3 and 24 h of immobilization) using different substrates at pH 7.0 and 37°C. Activity is given as increment of Absorbance at 280 nm after 15 minutes of reaction (see methods section).

Substrate	glyoxyl-ficin (1 h)	glyoxyl-ficin (3 h)	glyoxyl-ficin (24 h)
CS	0.45 ± 0.01	0.43 ± 0.02	0.38 ± 0.04
HG	0.35 ± 0.01	0.41 ± 0.05	0.42 ± 0.01
BSA	0.27 ± 0.03	0.27 ± 0.04	0.28 ± 0.03
BAPNA	0.88 ± 0.03	0.84 ± 0.03	0.71 ± 0.01

Table 3
Activity of different glyoxyl-ficin biocatalysts (1, 3 and 24 h of immobilization) using different substrates at pH 5.0 and 37°C. Activity is given as increment of Absorbance at 280 nm after 15 minutes of reaction (see methods section).

Substrate	glyoxyl-ficin (1 h)	glyoxyl-ficin (3 h)	glyoxyl-ficin (24 h)
CS	0.30 ± 0.01	0.28 ± 0.01	0.29 ± 0.01
HG	0.31 ± 0.04	0.30 ± 0.03	0.47 ± 0.01
BSA	0.24 ± 0.01	0.26 ± 0.03	0.25 ± 0.01
BAPNA	0.77 ± 0.01	0.76 ± 0.03	0.57 ± 0.01

immobilized in the support bearing the highest amount of HA, that increased the activity by almost a 3-fold factor, but remains 80 % of the activity of the Asp-ficin biocatalyst. This way, these biocatalysts were 1.8 fold more active versus CS than the free enzyme using the minimum amount of HA, or 1.68 fold with the maximum HA amount on the support. Using BSA as substrate at pH 7, the activity slightly decreased after immobilization, with scarce differences using different amounts of HA in the support (around 80 % of the activity using Asp-ficin). The modification of the enzyme and the support with glutaraldehyde to have enzyme-support covalent bonds[109] produced mixed results. The modification of Asp-ficin (no covalent bonds with the support can be formed in this case, just chemical modification and inter- or

Table 4
Activity of different biocatalysts of ficin prepared using Asp/HA supports, modified or not with glutaraldehyde, versus different substrates at pH 7.0 and 37°C. Activity is given as increment of Absorbance at 280 nm after 15 minutes of reaction (see methods section).

Substrate	Biocatalysts									
	Asp	Asp-GLU	25 mM HA	25 mM HA/ GLU	50 mM HA	50 mM HA/ GLU	100 mM HA	100 mM HA/ GLU	0.5 M HA	0.5 M HA/ GLU
CS	0.56 ± 0.04	0.73 ± 0.07	1.77 ± 0.13	0.44 ± 0.08	1.79 ± 0.02	0.44 ± 0.13	1.68 ± 0.11	0.49 ± 0.21	1.58 ± 0.16	0.5 ± 0.12
HG	0.58 ± 0.09	0.74 ± 0.02	0.16 ± 0.03	0.18 ± 0.12	0.15 ± 0.04	0.20 ± 0.04	0.16 ± 0.07	0.26 ± 0.06	0.46 ± 0.05	0.28 ± 0.01
BSA	0.33 ± 0.07	0.34 ± 0.03	0.25 ± 0.03	0.41 ± 0.02	0.26 ± 0.03	0.26 ± 0.01	0.71 ± 0.15	0.25 ± 0.02	0.27 ± 0.01	0.22 ± 0.05
BAPNA	0.52 ± 0.03	0.47 ± 0.02	0.32 ± 0.02	0.25 ± 0.01	0.30 ± 0.02	0.23 ± 0.01	0.28 ± 0.01	0.22 ± 0.01	0.33 ± 0.02	0.22 ± 0.01

intramolecular crosslinkings) improved the activity versus CS and HG (by 30 %), with scarce effect using BSA (increase by less than 5 %) and a slight decrease in the activity versus BAPNA (by 10 %) may be observed. When the support has HA, and covalent enzyme-support bonds also are possible, the results depend on the substrate used to determine the activity. Using BAPNA, the activity decreased by 22–25 % for all the Asp/HA biocatalysts except for the biocatalyst with maximum amount of HA, that reduced its activity to 66 %. The activity also decreased employing CS, to 25 % using the support with minimal amount of HA, slightly increasing with the amount of HA in the support (in contradiction with the results using the just ionic expanse immobilized biocatalysts). The biocatalyst prepared on the support with the highest amount of HA presented the highest activity (13 % more than the support with the lower amount of HA), decreasing the activity after glutaraldehyde modification to 32 %. With HG as substrate, the activity increased after the modification using the supports with lower amount of HA, and this effect increased with the amount of HA in the support, but experimented a drop in the activity for the support bearing the highest amount of HA (although it maintained the highest activity of the modified biocatalysts with this substrate, as the adsorbed enzyme was the most active one in this support). The use of BSA to determine the activity produced very different results. The activity increased by almost 1.5 fold for the Asp-HA bearing the lowest HA amount (becoming more active than the Asp-ficin and Asp-ficin-glutaraldehyde biocatalysts), and then progressively decreased when the amount of HA in the support increased (by almost 45 %). These results are fully different to the ones observed using glyoxyl-ficin or the free enzyme.

Analyzing the results obtained at pH 5 (Table 5), this pH promoted a decrease in the Asp-ficin biocatalyst activity using all the substrates (20 % of the activity at pH 7 using CS, 57 % using HG or to BSA, and 92 % using BAPNA). The different Asp/HA-ficin biocatalysts have mixed results depending on the substrate. The activity using CS significantly decreased in all cases after immobilization, increased using HG and had no relevant effects using BSA. Using BAPNA, a small increase in enzyme activity was found in all cases. That is, the enzyme action versus the different substrates seemed to be altered by the ionic exchange of the enzyme on these supports.

The glutaraldehyde treatment of the Asp-ficin biocatalysts promoted a decrease in the activity at this pH value using BAPNA as substrate (by 20 %). However, at this pH value, the activities versus all the substrate proteins increased after glutaraldehyde treatment, 1.90 folds using CS, 1.35 using HG and 1.14 using BSA. This suggested that the glutaraldehyde modification of the enzyme presented positive effects on the proteolytic activity of ficin. Using the Asp/HA supports, the modification also produced a moderate decrease of the activity versus BAPNA while the effects on the protease activity were mixed. Using the 2 M Asp/25 mM HA-ficin, the activity decreased to 62 % using CS and 82 % using BSA, while using HG the activity increased by more than 4.4-fold. The biocatalysts prepared on 2 M Asp/50 mM HA-ficin reduced the activity to 70 % versus casein, and increased the activity using BSA (by a 10 %) or HG (by a 2.1 fold factor). The 2 M Asp/100 mM HA-ficin biocatalyst increased its protease activity with all the protein substrates: to 152 % using CS, 250 % using HG and 330 % using BSA. This biocatalyst was more active versus BSA at pH 5 than the free enzyme even at pH 7 (Table 1), and multiplied by 3 the activity of the free enzyme at pH 5. This positive effect of the glutaraldehyde modification on the proteolytic activity versus all the studied protein substrates was also observed using 2 M Asp/500 mM HA biocatalyst; 137 % using CS, 289 % using HG and 270 using BSA. This biocatalyst was the most active using CS and HG at this pH value, and very similar to the 100 HA/Asp-ficin biocatalysts with BSA.

Obviously, the different and frequently opposed changes of activities versus different proteins altered the activities ratios of the biocatalysts using the different proteins. At pH 7, it can be remembered that the free enzyme exhibited a 0.7 value for HG and 0.4 for BSA. The Asp-ficin and Asp-ficin-glutaraldehyde biocatalyst showed very different ratios: 1 for HG (both biocatalysts) and 0.58 and 0.46 respectively. 25 mM HA/Asp-ficin gave values of 0.09 for CS and 0.13 for BSA, after glutaraldehyde modification this moved to 0.41 and 0.92 respectively. The 100 mM HA/Asp-ficin presented activity ratios of less than 0.09 for CS and 0.17 using BSA, that increased to 0.44 and 0.52, respectively, after glutaraldehyde modification. Using the 100 mM HA/Asp-ficin biocatalysts, the

Table 5
Activity of different biocatalysts of ficin prepared using Asp/HA supports, modified or not with glutaraldehyde, versus different substrates at pH 5.0 and 37°C. Activity is given as increment of Absorbance at 280 nm after 15 minutes of reaction (see methods section). GUU refers to biocatalysts treated with glutaraldehyde.

Substrate	Biocatalysts									
	Asp	Asp-GLU	25 mM HA	25 mM HA/ GLU	50 mM HA	50 mM HA/ GLU	100 mM HA	100 mM HA/ GLU	0.5 M HA	0.5 M HA/ GLU
CS	0.12 ± 0.06	0.23 ± 0.07	0.31 ± 0.08	0.19 ± 0.04	0.48 ± 0.06	0.33 ± 0.01	0.23 ± 0.03	0.50 ± 0.08	0.39 ± 0.03	0.55 ± 0.01
HG	0.32 ± 0.056	0.44 ± 0.07	0.20 ± 0.07	0.48 ± 0.06	0.25 ± 0.01	0.52 ± 0.07	0.26 ± 0.02	0.65 ± 0.07	0.26 ± 0.03	0.76 ± 0.08
BSA	0.19 ± 0.01	0.21 ± 0.05	0.17 ± 0.05	0.14 ± 0.01	0.27 ± 0.08	0.19 ± 0.07	0.17 ± 0.01	0.58 ± 0.04	0.18 ± 0.01	0.50 ± 0.01
BAPNA	0.48 ± 0.04	0.38 ± 0.03	0.34 ± 0.02	0.29 ± 0.03	0.29 ± 0.001	0.20 ± 0.01	0.31 ± 0.07	0.24 ± 0.02	0.36 ± 0.01	0.21 ± 0.02

activities ratios moved from 0.092 using CS and 0.15 for BSA to 0.53 and 0.60. Finally, using the biocatalysts with the highest amount of H, the values were 0.29 and 0.16, which increased to 0.56 and 0.44, for HG and BSA respectively. Only the biocatalyst prepared using this support presented a significantly difference in the activities rations before glutaraldehyde modification, mainly using HG, as a result of the increase in activity versus this substrate. The activities ratios presented important differences after the glutaraldehyde modification, increasing for the supports where covalent bonds may be formed and decreasing when the support is only Asp.

Next we have analyzed the activities ratios at pH 5. It should be reminded that the values were very different for the free enzyme at this pH compared to that at pH 7 (3.3 for HG and 0.91 for BSA. The enzyme immobilized in Asp- support presented values of activities ratio of 2.7 for HG and 1.57 (already very different to the values detected using the free enzyme). The modifications with glutaraldehyde decreased the ratio to 1.9 using HG and to 0.94 using BSA. Utilizing 25 mM HA/Asp-ficin, the values were 0.64 and 0.54, mainly by the increase in the activity versus CS. Its modification with glutaraldehyde caused the activities ratios to change from 2.5 to 0.72, mainly by the decrease in the activity versus CS. The 50 mM HA/Asp-ficin biocatalyst gave an activities ratio of 0.52 for HG and 0.36 for BSA, increasing to 1.56 and 0.57, respectively. The 100 mM HA/Asp-ficin biocatalysts gave activities ratios of 0.78 and 0.52, for HG and BSA, which were transformed to 1.28 and 1.14, respectively by the glutaraldehyde modification (activities with the 3 proteins became quite similar). Finally, 500 mM HA-Asp-ficin gave values of 0.66 (that moved to 1.4 after glutaraldehyde modification) with HG and 0.46 (0.38 after modification) with BSA.

That way, the immobilization on the different support and the glutaraldehyde modification greatly altered the substrate specificity of the immobilized ficin biocatalysts.

4. Conclusion

The results showed in this paper confirm that the different modification of proteases induced during its immobilization can alter the protein substrate specificity, considering that the protein substrate will remain identical, these changes should be related to enzyme distortion, that makes the enzyme to prefer one protein over another. We have not been able to provide physical evidences of these changes due to the use of a ficin crude extract. Also the response to change in the pH value depends on the employed immobilization protocol. This way, protease immobilization becomes a potent tool to alter this enzyme feature.

On the other hand, it is also clear that some changes in the ficin structure that are detrimental when using a substrate-protein, may become positive using other substrate protein (in some instances activity is higher than using the free enzyme). This way, the properties of the immobilized protease should be analyzed utilizing exactly the protein (or protein extract) that we intend to hydrolyze, as extrapolate the data with one protein to the activity versus other protein is a not straightforward process.

CRediT authorship contribution statement

Roberto Fernandez-Lafuente: Writing – review & editing, Writing – original draft, Supervision, Formal analysis, Data curation, Conceptualization. **El Siar Hocine:** Writing – original draft, Investigation, Formal analysis, Data curation. **Alex D. G onzalez-Vasquez:** Writing – review & editing, Writing – original draft, Investigation, Data curation. **Javier Rocha-Martin:** Writing – review & editing, Writing – original draft, Formal analysis, Conceptualization. **Marcela Urzúa:** Writing – review & editing, Supervision.

Data Availability

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

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Authors agreement

All authors certify that they have seen and approved the final version of the manuscript being submitted entitled "**Changes in ficin specificity by different substrate proteins promoted by enzyme immobilization**". We warrant that the article is the authors' original work, hasn't received prior publication and isn't under consideration for publication elsewhere.

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