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Proteases of fig: extraction, immobilization and application

Protéases de figuier : extraction, immobilisation et application

Submitted by El Hocine SIAR

Board of Examines

Chairman: Prof. Leila BENATALLAH. Mentouri Brothers University, Constantine -1-

Supervisor: Prof. Mohamed Neseredine ZIDOUNE. Mentouri Brothers University, Constantine -1-

Co- Supervisor: Prof. Roberto FERNANDEZ LAFUENTE Instituto de Catálisis y Petroleoquímica, UAM-CSIC, Cantoblanco, Madrid, Spain

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HOCINE

Dedication

With my deepest feelings of gratitude, I dedicate this modest work

To my dear parents Kaci and Zina, without them I could'nt be what I am, in recognition of their efforts, love and encouragement.

To my beloved Nacera, as a sign of love and gratitude for having supported and above all understood me permanently, these encouragements and his kindness.

To my dear brother Lahcen and her wife and children: Akcel, Makhlouf, Celine and Ania.

To my Sister Fatma and her husband Hamou and children: Aya, Israe and Oussama

To my Sister Malika and her husband Djamel and children: Rayen end Ayla

To my brothers Boudjamaa and Idir

To my little sister Wardia

To all my family, friends and everyone who loves me.

I love you so much

CONTENT

LIST OF ABBREVIATION, FIGURES AND TABLES

List of abbreviation

EDTA: Éthylènediaminetétraacétic

TCA: trichloroacetic acid

NaOH: sodium hydroxide

$NaBH_4$: sodium tetrahydruroborate

NaIO4: sodium periodate

BANA: Benzoyl-arginine-p-nitroanilide

EDA: ethylenediamine

EDC:1-Ethyl-3-(3-dimethylaminopropyl)-Carbodiimide

kD: kilo Dalton

PDB: Protein Data Bank

Arg: argenin

Lys: lysin

Glu : glutamic acid

Asp : aspartic acid

3D: three dimensión

NaCl: sodium clorure

mM: mili molar

mg: mli gran

ABT: Agarose Bead Technologies

List of figures and tables

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I. Introduction

Human beings started using enzymes long before they were described, in processes like wine, beer or cheese making (FERNANDES, 2019 and MAY, 2019). Nowadays, enzymatic biocatalysis has become a very adequate solution to reach the goals raised by the regulations of green chemistry (BINOD, *et al.,* 2019). They are very active under mild temperature and pressure conditions, very selective (giving just one product among several possible ones), and specific (recognizing just one substrate in a mixture of similar compounds). However, since they are designed by nature to give rapid answer to changes in the environment, enzymes have some limitations as industrial catalysts: they are water-soluble, relatively unstable, inhibited by different compounds and their properties are exhibited versus their natural substrate under physiological conditions (PORTER, *et al.,* 2016). Nowadays, there are many solutions to overcome these limitations, using genetic (metagenomics, site-directed mutagenesis or directed evolution), physicochemical tools (chemical or genetic modification) or even reactor design (PORTER, *et al.,* 2016). Among enzymes, proteases are an outstanding example of this long-standing use of biocatalysis by mankind. These enzymes are present in all living organisms and have found applications in diverse industrial areas (TAVANO, *et al.,* 2018).

In some instances, chemical alternative exists (CUQ *et al.,* 1983) but proteases have clear advantages, as the process will be more specific, avoiding the production of by-products or destruction of some amino acids. 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 (MASUDA *et al.,* 2010), pharmaceutical (FEIJOO-SIOTA, *et al.,* 2011) and in cheese making (SHAH, *et al.,* 2014, and FACCIA, *et al.,* 2012) industries. It has also been used for bioactive peptides production (ESMAEILPOUR, *et al.,* 2016 and DI PIERRO *et al.,* 2014) and meat tenderization (BEKHIT, e*t al.,* 2014 and SULLIVAN *et al.,* 2010).

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, (MARIANT, *et al.,* 1991, SHAM *et al.,* 2014 and SACZYNSKA, *et al.,* 2018). Four isoforms of ficin have been described (A, B, C and D) and crystallized, resolving their structure (HAESAERTS *et al.,* 2015 and ZARE *et al.,* 2013). Ficin isoforms are glycoproteins and present a high sequence similarity with bromelain (DEVARAJ, *et al.,* 2008).

In Algeria, fig tree latex containing a protease activity, called ficin was traditionally used as milk clotting agent particularly in Kabylie to produce a traditional cheese known as "Agugli" or "Igisi" according to the region.

Proteases are relatively expensive and the reuse of the enzymes seems necessary to get economically suitable processes (TAVANO, 2013). The immobilization of an enzyme may facilitate enzyme reuse and that way, the economic feasibility of the process (DI COSIMO *et al.,* 2013, CANTONE *et al.,* 2013, SHELDON and VAN PELT 2013, LIESE, and HILTERHAUS, 2013). Protease immobilization is the most suitable tool to facilitate its reuse provided that the immobilized enzyme is stable enough (SHELDON, and VAN PELT,2013, and TRAN, and. BALKUS 2011). Considering this fact, it seems adequate to couple the improvement of other enzyme features to its immobilization. Thus, an intense research in this area has been developed and has shown that a proper immobilization may have some further positive effects on many enzyme features. Immobilized enzymes have in some instances an improved activity, selectivity or specificity due to conformational changes caused by immobilization (SECUNDO, 2013) or due to enzyme stabilization that permit to keep the enzyme conformation under more drastic conditions, preventing aggregation, unfolding, etc. (RODRIGUES, *et al.,* 2013). In some instances, enzyme immobilization may be coupled to enzyme purification (BARBOSA, *et al.,* 2015). immobilization may be used to improve other enzyme limitations, like resistance to chemicals or inhibitors, even purity (MATEO, *et al.,* 2007, BARBOSA, *et al.,* 2015 and SANTOS, *et al.,* 2015).

In the case of proteases, immobilization on porous supports will have both additional problems and advantages compared to other enzymes. A clear advantage is that an immobilized protease molecule cannot be attacked by other immobilized protease molecule (this protection does not occur using non-porous materials). Thus, inactivation by enzyme proteolysis may not occur (GARCIA-GALAN *et al.,* 2011 and CIPOLATTI *et al.,* 2016).

Today, biotechnology is oriented towards the search for the most reliable means to ensure the availability and stability of proteases. It is in this perspective that my thesis is inscribed. The objectives of this work were the preparation of an immobilised biocatalyst of ficin with improved characteristics (activity, thermal and chemical stability and stabilization in presence of chemical agents) compared to the free enzyme and for an eventual reuse of this biocatalysts in the hydrolysis of proteins (in our case we used a bovine casein).

1- Bibliographical part which summarizes the characteristics of ficin, use of ficin, immobilization of ficin,

2- The presentation of different results obtained during from our investigation, this in the form of an article starting with the extraction and characterization of the immobilization and the chemical modification at the end of the application of the obtained biocatalysts;

3- Finally, a general conclusion on all the work.

INTRODUCTION

Les humains ont commencé à utiliser des enzymes bien avant leur description, dans des processus tels que la fabrication du vin, de la bière ou du fromage (FERNANDES, 2019 et MAI , 2019) . De nos jours, la biocatalyse enzymatique est devenue une solution très adéquate pour atteindre les objectifs de la chimie verte (BINOD, et al., 2019) . Ils sont très actifs dans des conditions de température et de pression, très sélectifs (ne donnant qu'un seul produit parmi plusieurs possibles) et spécifiques (ne reconnaissant qu'un seul substrat dans un mélange de composés similaires). Cependant, comme ils sont conçus par nature pour donner une réponse rapide aux changements de l'environnement, les enzymes ont certaines limites en tant que catalyseurs industriels : ils sont solubles dans l'eau, relativement instables, inhibés par différents composés et leurs propriétés sont exposées par rapport à leur substrat naturel sous des conditions physiologique. (PORTER , et al., 2016). Maintenant, il existe de nombreuses solutions pour maîtriser ces désavantages, en utilisant des outils génétiques (métagénomique, mutagenèse dirigée ou évolution dirigée), physico-chimiques (modification chimique ou génétique) ou encore la conception de réacteurs (PORTER, et al., 2016). Parmi les enzymes, les protéases sont un exemple remarquable de cette utilisation de longue date de la biocatalyse par l'humanité. Ces enzymes sont présentes dans tous les organismes vivants et ont trouvé des applications dans divers domaines industriels (TAVANO , et al., 2018) .

Dans certains cas, des alternative chimique sont utilisée (CUQ et al., 1983) mais les protéases présentent des avantages évidents, car le procédé sera plus spécifique, évitant la production de sous-produits ou la destruction de certains acides aminés . Parmi les protéases d'origine végétale les plus utilisées, le latex du figuier (qui contient la ficine). Il a été utilisé dans l'industrie de brassage (MASUDA et al., 2010) , pharmaceutique (FEIJOO - SIOTA , et al., 2011) et dans la fabrication du fromage (SHAH , et al., 2014 , et FACCIA , et al., 2012). Il a également été utilisé pour la production de peptides bioactifs (ESMAEILPOUR , et al., 2016 et DI PIERRO et al., 2014) et l'attendrissement de la viande (BEKHIT , et al., 2014 et SULLIVAN et al., 2010) .

La ficine est considérée pour la répétabilité des résultats d'hydrolyse dans la génération d'anticorps via l'hydrolyse spécifique de certaines liaisons peptidiques, (MARIANT , et al., 1991 , SHAM et al., 2014 et SACZYNSKA , et al., 2018) . Quatre isoformes de la ficine ont été identifiées (A, B, C et D) cristallisées, (HAESAERTS et al., 2015 et ZARE et al., 2013) . Les isoformes de la ficine sont des

glycoprotéines qui présentent une similitude de séquence élevée avec la bromélaïne (DEVARAJ , et al., 2008).

En Algérie, le latex de figuier contenant une enzyme protéolytique, appelée ficine était traditionnellement utilisé comme agent de coagulation du lait en particulier en Kabylie pour produire un fromage traditionnel connu sous le nom « AGUGLUI » ou « IGISI » selon la région.

Les protéases sont relativement chères et la réutilisation des enzymes semble nécessaire pour obtenir des procédés économiquement adaptés (TAVANO, 2013). L'immobilisation d'une enzyme peut faciliter la réutilisation de l'enzyme et ainsi, la faisabilité économique du procédé (DI COSIMO et al., 2013, CANTONE et al., 2013, SHELDON et VANPELT 2013, LIESE et HILTERHAUS, 2013). L'immobilisation de la protéase est l'outil le plus approprié pour faciliter sa réutilisation à condition que l'enzyme immobilisée soit suffisamment stable (SHELDON et VAN PELT, 20 13, et TRAN, et. BALKUS 2011). Compte tenu de ce fait, il semble adéquat de coupler l'amélioration d'autres caractéristiques de l'enzyme à son immobilisation. Ainsi, une recherche intense dans ce domaine a été développée et a montré qu'une immobilisation appropriée peut avoir d'autres effets positifs sur de nombreuses caractéristiques enzymatiques. Les enzymes immobilisées ont dans certains cas une activité, une sélectivité ou une spécificité améliorée en raison de changements conformationnels provoqués par l'immobilisation (SECUNDO, 2013) ou en raison de la stabilisation enzymatique qui permettent de maintenir la conformation enzymatique dans des conditions plus drastiques, empêchant l'agrégation, le déploiement, etc. (RODRIGUES, et al., 2013). Dans certains cas, l'immobilisation des enzymes peut être couplée à la purification (BARBOSA, et al., 2015). L'immobilisation peut être utilisée pour améliorer d'autres caractéristiques enzymatiques, comme la résistance aux produits chimiques ou aux inhibiteurs, voire la pureté de l'enzyme (MATEO, et al., 2007, BARBOSA, et al., 2015 et SANTOS, et al., 2015).

Dans le cas des protéases, l'immobilisation sur des supports poreux présente à la fois des problèmes et des avantages par rapport à d'autres enzymes. Un avantage clair est qu'une protéase immobilisée ne peut pas être attaquée par une autre protéase immobilisée (cette protection ne se produit pas en utilisant des matériaux non poreux). Ainsi, l'inactivation par

protéolyse enzymatique peut ne pas se produire (GARCIA - GALAN et al., 2011 et CIPOLATTI et al., 2016).

Aujourd'hui, la biotechnologie est orientée vers la recherche des moyens les plus fiables pour assurer la disponibilité et la stabilité des protéases. C'est dans cette perspective que s'inscrit notre travail. Il vise à apporter de nouvelles méthodes de stabilisation de la ficine pour une utilisation plus rationnelle et une possibilité de son utilisation et de sa réutilisation dans l'hydrolyse des protéines dans différentes conditions, pour cela nous avons procédé à l'immobilisation sur de l'agarose activé (glyoxyl ou amino ou glutaraldéhyde), ce manuscrit est composé de trois parties principales :

1. *Une partie bibliographique qui résume les caractéristiques, utilisations et immobilisation de la ficine ;*
2. *Présentation des résultats obtenues sous forme d'articles, en commençant par l'extraction et la caractérisation, l'immobilisation et les modifications chimique et en fin application de biocatalyseur obtenues ;*
3. *Et enfin, conclusion générale*

BIBLIOGRAPHY

This part is presented by a scientific publication as a bibliographical review with the title *" Ficin: A protease extract with relevance in biotechnology and biocatalysis"* with the aim of summarizing the studies that have been carried out on the ficin and its applications.

This review aims to summarize the recent uses of ficin, for example in protein hydrolysis, production of bioactive peptides and antibodies fragments, tenderization, milk coagulations in cheese making or peptide synthesis. Immobilization and production of biocatalyst of immobilized ficin will be also described.

Abstract

Due to the problems raised by the use of animal or microbial recombinant proteases, the use of proteases from vegetable origin is becoming increasingly popular. Among them, sulfidryl proteases have a special interest. Ficin is an outstanding example of this kind of proteases. This paper aims to be to make a comprehensive review of the recent uses of this enzyme, including for example protein hydrolysis, the production of bioactive peptides and antibodies fragments (researchers point that ficin results are more reproducible than using other proteases), meat tenderization, milk coagulations in cheese making or peptide synthesis. Efforts to get industrial immobilized biocatalysts of the enzyme will be also described. The review shows the huge potential and brilliant prospect that this enzyme can have in the near future.

Keywords: Proteases, Proteolysis, Synthesis of peptides, Bioactive peptides, Protease immobilization.

Resumé

En raison des inconvénients engendrés par l'utilisation de protéases animales ou microbiennes, l'utilisation de protéases d'origine végétale est de plus en plus encouragée. Parmi elles, les protéases à cystéine présentent un intérêt particulier. La ficine est un exemple de ce type de protéases. Cet article vise à faire un résumé complet de récentes utilisations de cette enzyme par exemple, dans l'hydrolyse des protéines, la production de peptides bioactifs et de fragments d'anticorps (des chercheurs ont conclu que les résultats obtenus avec la ficine sont plus reproductibles que ceux obtenues avec d'autres protéases), l'attendrissement de la viande, coagulations du lait et fabrication du fromage ou la synthèse peptidique. Les récentes avancées sur l'immobilisation de la ficine et la production des biocatalyseurs industriels seront également décrits. Cette recherche montre l'importance que cette enzyme peut avoir dans un proche avenir.

Mots clés : *Protéases, Protéolyse, Synthèse de peptides, Peptides bioactifs, Immobilisation de protéases.*

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Review

Ficin: A protease extract with relevance in biotechnology and biocatalysis

Roberto Morellon-Sterling [a,1], Hocine El-Siar [a,b,1], Olga L. Tavano [c], Ángel Berenguer-Murcia [d], Roberto Fernández-Lafuente [a,1,*]

[a] Departamento de Biocatálisis, ICP-CSIC, Campus UAM-CSIC, 28049 Madrid, Spain
[b] Equipe TEPA, Laboratoire LNTA, INATAA, Université des Frères Mentouri Constantine 1, 25000 Constantine, Algeria
[c] Alfenas Federal University, Faculty of Nutrition, 700 Gabriel Monteiro da Silva St, Alfenas, MG 37130-000, Brazil
[d] Departamento de Química Inorgánica e Instituto Universitario de Materiales, Universidad de Alicante, Alicante 03080, Spain

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abstract

Due to the problems raised by the use of animal or microbial recombinant proteases, the use of proteases from vegetable origin is becoming increasingly popular.. Among them, sulfidryl proteases have a special interest. Ficin is an outstanding example of this kind of proteases. This paper aims to be to make a comprehensive review of the recent uses of this enzyme, including for example protein hydrolysis, the production of bioactive peptides and antibodies fragments (researchers point that ficin results are more reproducible than using other proteases), meat tenderization, milk coagulations in cheese making or peptide synthesis. Efforts to get industrial immobilized biocatalysts of the enzyme will be also described. The review shows the huge potential and brilliant prospect that this enzyme can have in the near future.

Contents

* Corresponding author at: ICP-CSIC, C/ Marie Curie 2, Campus UAM-CSIC, Cantoblanco, 28049 Madrid, Spain.
E-mail address: rfl@icp.csic.es (R. Fernández-Lafuente).
[1] Both authors have evently contributed to this paper

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1. Introduction

Human beings started using enzymes long before they were described, in processes like wine, beer or cheese making [1,2]. Nowadays, enzymatic biocatalysis has become a very adequate solution to reach the goals raised by the regulations of green chemistry [3]. They are very active under mild temperature and pressure conditions, very selective (giving just one product among several possible ones), and specific (recognizing just one substrate in a mixture of similar compounds). However, since they are designed by nature to give rapid answer to changes in the environment, enzymes have some limitations as industrial catalysts: they are water-soluble, relatively unstable, inhibited by different compounds and their properties are exhibited versus their natural substrate under physiological conditions [4]. Nowadays, there are many solutions to overcome these limitations, using genetic (metagenomics, site-directed mutagenesis or directed evolution), physicochemical tools (chemical or genetic modification) or even reactor design [4].

Among enzymes, proteases are an outstanding example of this long-standing use of biocatalysis by mankind. These enzymes are present in all living organisms and have found applications in diverse industrial areas [5]. For example, in food biotechnology (e.g., altering the protein matrices that constitute most foods) they are used to alter sensorial qualities (such as flavor or texture), improve digestibility, reduce allergenicity, produce bioactive peptides, etc. [6–8]. The remnant hydrolysis of the κ casein fragment in milk casein is the first step in milk aggregation, the starting point of cheese production and one of the oldest protease applications [9]. One of the main current applications of proteases is in detergent formulations. Proteases have also found some roles in pharmaceutical industries, e.g., to produce dipeptides from amino acids or in the resolution of racemic mixtures [10,11].

1.1. Vegetable proteases: *ficin*

Among proteases, vegetable proteases have attracted a great interest, mainly in food applications [12–16]. Consumers showed some rejection to proteases from animal sources [17] by the risks of illness transmission, while recombinant proteases cannot be utilized in human foods in certain countries. In this sense, ficin is an enzyme extract composed by several proteases that is attained from the latex of the fig (Ficus carica). The exact relation of the different active components of ficin extract may change by the health of the tree, the ambient conditions, watering, etc. [18,19]. For example, latex presents a uniform increase of protein concentration during fruit ripening, while the content of ficin decreased. [20]. Ficin forms with different specificities are present in different proportions during fruit ripening, Four proteases have been crystallized to date (A, B, C and D) and their structures are available [18,21]. Their analysis reveals that all four are glycoproteins, presenting a high sequence similarity to bromelain [21].

In a rapid view, some efforts to purify ficin extract and identify its main components may be summarized as follows. Efforts to purify and analyze the composition and function of ficin started just after it was reported. Ficin was purified by several precipitations with ammonium sulfate followed by gel filtration using Sephadex G-100 [22]. Ficin and other protein fractions were obtained.

The active thiol group of ficin has been marked with N-(4-dimethylamino 3,5-dinitrophenyl) (DDPS)-maleimide, and submitted to pepsin hydrolysis [23]. The peptide containing DDPS-cysteine was Tyr-Ser-Gly-Val-DDPS-Cys. The N-terminal residue of this ficin form was leucine. The amino acid composition of ficin has been determined [23] (see table IS), showing a great diversity among the different isoforms. Ficin has been isolated from commercial preparations by salt fractionation and chromatography on carboxymethyl cellulose. Although several ficin forms were found, only the main component was characterized. It was found that using this protease, a wide variety of peptide bonds were hydrolyzed, but peptide bonds following an aromatic residue seemed to be hydrolyzed more efficiently than the others. That is, the enzyme has a preference for aromatic residues, but it was quite unspecific [24]. Later on, another paper proposed the use of agarose-mercurial column to purify ficin from Ficus carica, enabling to separate ficin and mercurificin [25]. In another paper, a glycosylated proteinase from Ficus carica var. Hōraishi was purified by CM-Sephadex C-50 and CM-cellulose, and called Ficin S. The purified Ficin S was electrophoretically homogeneous [26]. The optimal pH of the enzyme was pH 8.0, and optimal temperature was 60 °C. The enzyme was described to be stable at a pH range 2.0–8.0 at 4 °C for 20 h. The enzyme activity increased in the presence of cysteine and mercaptoethanol, but it was inhibited by p-chloromercuribenzoate and $HgCl_2$. This enzyme could be added to ficin A, B, C and D from Ficus carica var. Hōraishi. The results suggest that Ficin S differs only in isoelectric point and sugar content from the other ficin isoforms. The PDB code for some of the enzymatic components of ficin is listed below. For Ficin A, it is code 4YYQ, for ficin B it is code 4YYR, for Ficin C is code 4YYV and for Ficin D2, it is code 4YYW.

More recently, cultures of Ficus carica cells were used to produce ficin. These cultures presented an optimal activity versus benzoyl-arginine-p-nitroanilide (BAPNA) after 28 days, The enzyme was purified by high performance gel filtration [27]. The chromatography using thiopropyl Sepharose 6B was able to retain around 50% of the total activity, suggesting that it was due to thiol-proteins. Other sources of ficin have been analyzed. For example, fig latex from Ficus glabrata presented at least six ficin components [28]. Three ficins were separated and purified by salt precipitation, chromatography on CM-cellulose, and gel filtration. Two crystalline ficins (II and III) were obtained and they were homogeneous. $HgCl_2$ inhibited the enzymes. Autolysis did not seem to be a problem in ficin handling. Both enzymes have different amino acid composition [28].

The physiological role of ficin remains under debate. It has been suggested that some proteases in plants may be related to protection versus parasites. Leaf latex of some laticiferous plants, (e.g., Ficus virgate) presented growth inhibition and toxicity versus lepidopteran larvae [29]. This effect was correlated to cysteine proteases, such as papain, ficin, or bromelain, suggesting that they can be a general defence mechanism against herbivorous insects. In another paper, the authors suggested that proteases (including ficin) are related to the regulation of the recognition of pathogens and pests and the induction of effective defence responses in plants. [30].

In some instances, ficin has a negative impact on the properties of the plant derivatives for human consumption. Plant cysteine proteases have been related, at least partially to the cross allergy generated by fruits from different plants, being ficin one of the enzymes with highest impact [31].

Ficin cocktail applications are growing steadily, becoming one of the most used vegetable enzymes even though the specificity and mechanism of action of all the components are not fully characterized. Even after some long-standing applications, there is not a review paper listing the uses of this enzyme extractThis review intends to show the different applications of this very interesting protease extract and how it has been prepared to become an industrial biocatalyst, including its immobilization.

2. Ficin immobilization

The initial main goal of enzyme immobilization was to solve the problem of enzyme solubility in aqueous medium, as enzymes were very expensive biocatalysts [32]. Nowadays, the price of some commercial enzymes has gone down and some companies supply enzymes to be used in free form in processes like biodiesel production [33–37]. However, enzyme immobilization has become a tool with many more applications than mere reuse of enzymes. A proper immobilization can greatly improve many enzyme features [38–47]. Stability may be improved via immobilization by different causes [47]. First, using a porous

support most enzymes will be protected from outsider interfaces or intermolecular phenomena using any immobilization protocol (e.g., aggregation or proteolysis) [45]. This is especially relevant in the case of proteases like ficin because proteolysis may play a relevant role in the enzyme inactivation [5]. Second, immobilization via multipoint covalent attachment to rigid supports may increase the rigidity of the enzyme structure, reducing any conformational changes an increasing enzyme stability and increasing the range of conditions where the enzymes may be utilized [45]. Finally, if the enzyme is multimeric, the immobilization of all enzyme subunits may prevent enzyme dissociation [48]. The use of physically active supports (mainly hydrophobic ones) may even have negative effects on enzyme stability driving to enzymes with lower stability than its free counterparts [49]; also the distortion caused by the immobilization may expose groups sensible to oxidation or other chemical modification, also leading to a decrease in enzyme stability. An increase in enzyme stability can not only extend the useful lifetime of the enzyme in the reactor, it may also expand the range of conditions where the enzyme is utilized and thus increase the prospects of reaching an industrial-level process [48,50–53]. However, the real view of the enzyme stability in an industrial reactor should be better measured in terms of the mass of products produced by the mass of biocatalysts more than in terms of half-lives [54]. In this context, enzyme immobilization may increase the enzyme activity, in some instances by really fixing a more active enzyme form (e.g., in the case of the lipases immobilized via interfacial activation) [55], in some other by making the enzyme more resistant to unfavorable conditions [48,50–53], etc. This higher activity will mean following this idea, an "increased stability" as the enzyme will produce more product before being discarded. The multiple effects of immobilization on enzyme activity have been reviewed, listing facts and artifacts that can alter the enzyme activity [44]. Immobilization may be also utilized to tailor enzyme specificity, selectivity, resistance to inhibitors, etc. [47]. These changes are not so easy to predict, the aim is to build a library of very different immobilized biocatalysts of the enzymes and, that way, to increase the prospects of getting a favorable immobilized biocatalyst for a specific process [48,50–53]. Finally, a properly designed immobilization may also permit the specific immobilization of a target enzyme, coupling enzyme immobilization to its purification [56].

Immobilization of proteases may have an additional problem not relevant for other enzymes acting versus small substrates. As one their main applications is the hydrolysis of proteins, which are large substrates, only properly oriented immobilized enzyme molecules will maintain enzyme activity [5] (Fig. 1). A lowly loaded protease biocatalyst may have good activity versus proteins even if its orientation regarding the support surface is not perfect and the active center is not fully oriented opposite to this support surface. In this instance, the only requirement to give some activity is to have the enzyme active center far enough from the support surface [44]. An increase in the enzyme load increases the demand for a proper orientation, an immobilized protease biocatalyst where the support is fully coated with enzyme molecules will remain active only if the enzyme molecules are almost perfectly oriented opposite to the support surface [44] (Fig. 2).

Although ficin is mainly used in free form, it has been immobilized using different protocols. The first report on ficin immobilization may be found in 1976, showing the covalent immobilization of the enzyme [57]. Later on, bromelain, ficin, papain, pepsin and trypsin were immobilized on two different cellulose supports: cyanogen bromide or glutaraldehyde [58].Cyanogen bromide-cellulose gave better results than glutaraldehyde-cellulose, giving higher activities and stabilities. In another research, ficin was covalently immobilized on paper sheets bearing carboxymethyl groups via the carbodiimide route or Woodward Reagent K [59]. The paper sheets bearing immobilized enzymes exhibited acceptable enzyme activities together to suitable mechanical and brightness features. In another paper, papain, ficin, and bromelain were immobilized on chitosan beads, using glutaraldehyde or other activating agents [60]. The immobilized enzymes were quite active versus the synthetic and small substrate N-benzyl-L-arginine ethyl ester while activity was very low using casein, very likely due to steric reasons. Although the enzymes activities were lower after immobilization, the pH, thermal, and storage stability increased upon immobilization and the resulting catalysts could be used in a continuous way without detecting losses in enzyme activity [60]. Later, the same group immobilized

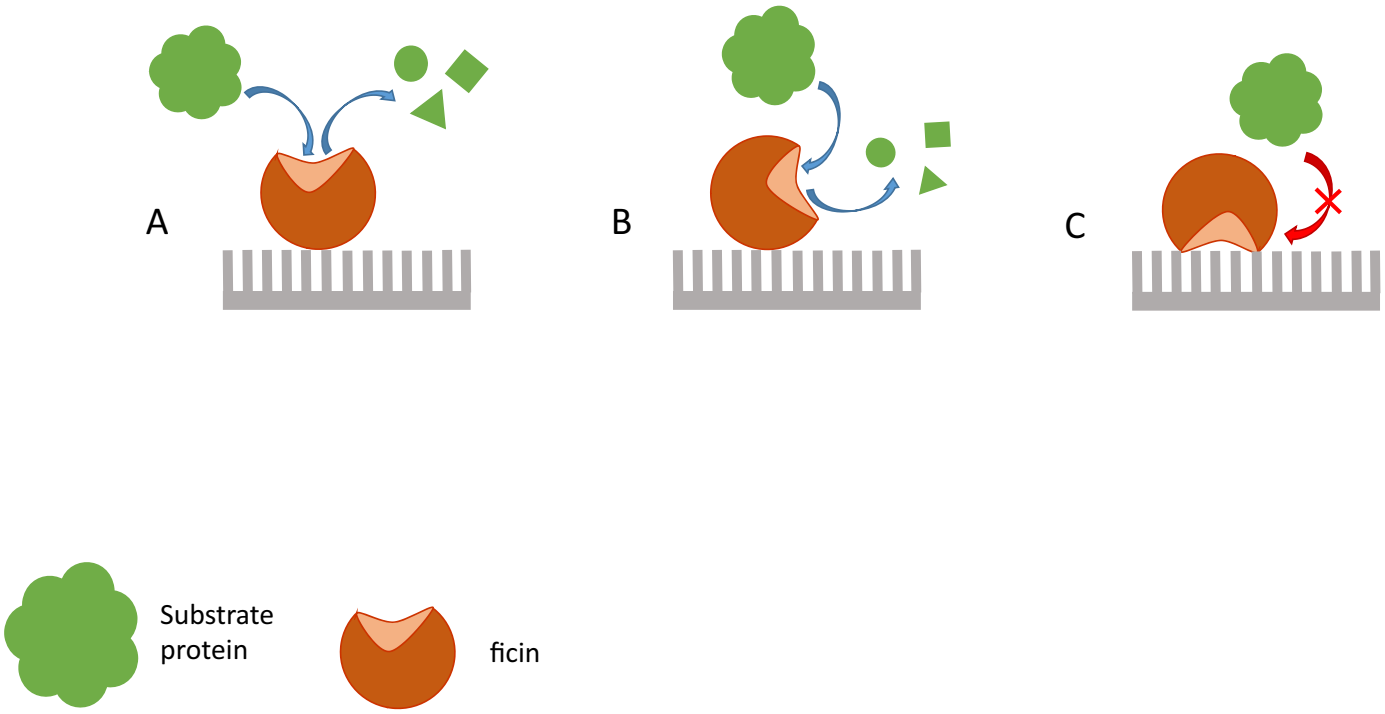

Fig. 1. Effect of the protease immobilization orientation on the activity of proteases versus large substrates.

papain, ficin, and bromelain onto the porous poly(vinyl alcohol) beads activated with hexamethylene diisocianate (HMDI) or cyanogen bromide [61]. Results in terms of activity were similar: high activity recovery versus N-benzyl-L-arginine ethyl ester and very low activity using casein. Stability was also improved. Using hexamethylene diisocianate the expressed activity did not depend on the enzyme loading while when using cyanogen bromide the relative activity decreased when the enzyme loading increased [61]. In another research effort, α-chymotrypsin, trypsin, bromelain, and ficin were immobilized on five different enzyme-membrane systems [62]. While using chymotrypsin high self-sustained pH oscillations were observed in a flat membrane, this only occurred with the other enzymes under specific conditions. Other research reports described how papain and ficin were covalently immobilized on poly (γ-methyl-D-glutamate) fibers [63]. The expressed activity of the immobilized enzyme versus N-benzyl-L-arginine ethyl ester was high, although Km was higher and Vmax was smaller. This could be compensated by the higher stability of the immobilized enzymes, and the possibility of using them in a continuous way [63]. Ficin has been also immobilized in poly (vinyl alcohol) electrospun nanofibers by crosslinking with glutaraldehyde vapor [64]. The optimal biocatalysts retained 92% of the enzyme activity versus N_{α}-benzoyl-L-arginine-4-nitroanilide hydrochloride and could be used in 9 cycles maintaining their activity. Immobilization may protect not only versus heat or pH, but also versus UV irradiation. The increase of free ficin globule size accompanied by a decrease in its activity was found after exposure to a radiation of 3020 $J \cdot m^{-2}$ intensity [65]. Enzyme immobilization on chitosan produced an increase in the stability in the presence of UV irradiation, suggesting that chitosan can behave as photoprotector.

In some instances, the key point is enzyme application. In order to use the enzymes in organic media, immobilization is convenient to increase enzyme stability and prevent aggregation [45]. Thus, ficin and papain were immobilized and utilized as biocatalysts in the synthesis of glutamine-, tyrosine and cystine-containing dipeptides using N-protecting groups in organic medium [66]. Later, ficin was trapped in starch beads and used in the kinetically controlled synthesis of peptides using N-protected amino acids and amino acid esters [67].

Ficin has also been immobilized via physical adsorption on Celite, and used in milk coagulation to produce teleme [68]. The product obtained using the immobilized enzyme presented better chemical and sensory features than those obtained using the free enzyme. Immobilized ficin (unfortunately, the paper did not give any detail on the immobilization support nor the followed strategy) was utilized to produce mini anti-growth factor receptor-vIII rom, a monoclonal antibody to increase circulation, retention, and enhance permeability [69]. In a quite unexpected application of ficin, glucose oxidase and ficin (using a peroxidase-like activity of this enzyme extract) were used to detect glucose [70]. The enzymes were immobilized on SiO_2@Fe_3O_4 nanoparticles. Later on, ficin was immobilized in an enzyme-metal organic framework composite with 2-methylimidazole and $zinc^{2+}$ ions, increasing by 2.5 fold the peroxidase activity [71]. This biocatalyst was coupled to glucose oxidase to detect glucose. In another paper, a Surface Plasmon Resonance Imaging sensor was designed using ficin, chymopapain or bromelain to detect cystatin in saliva, blood plasma and, urine [72]. To reach this goal, the enzymes were immobilized in a gold chip coated with cysteamine, activating the enzyme by treatment with N-Ethyl-N′-(3-dimethyl aminopropyl) carbodiimide/N-hydroxysuccinimide.

Our group has been quite active on ficin immobilization. The enzyme extract was first immobilized on glyoxyl agarose beads [73]. The optimal biocatalysts enhanced the enzyme stability by 40-folds at pH 5 and have 60% activity versus casein and benzoyl-D,L-arginine p-nitroanilide hydrochloride substrates. This biocatalyst retained twice the activity of the free enzyme at pH 10 and triplicated the activity retention at 80 °C). The proteolytic activity in the presence of 2 M of urea of the immobilized enzyme was 3-folds higher than that of the free enzyme [73]. The results were interesting, but still far from those obtained using other enzymes and glyoxyl support. To improve the stabilization, the enzyme was chemically aminated following the carbodiimide route before immobilization. That way a higher enzyme-support multipoint covalent attachment may be expected [74,75]. First, it was checked that the amination does not have a significant effect on immobilized enzyme activity or stability [76]. Then, the enzyme was aminated in free form, retaining around 80% of the initial activity versus

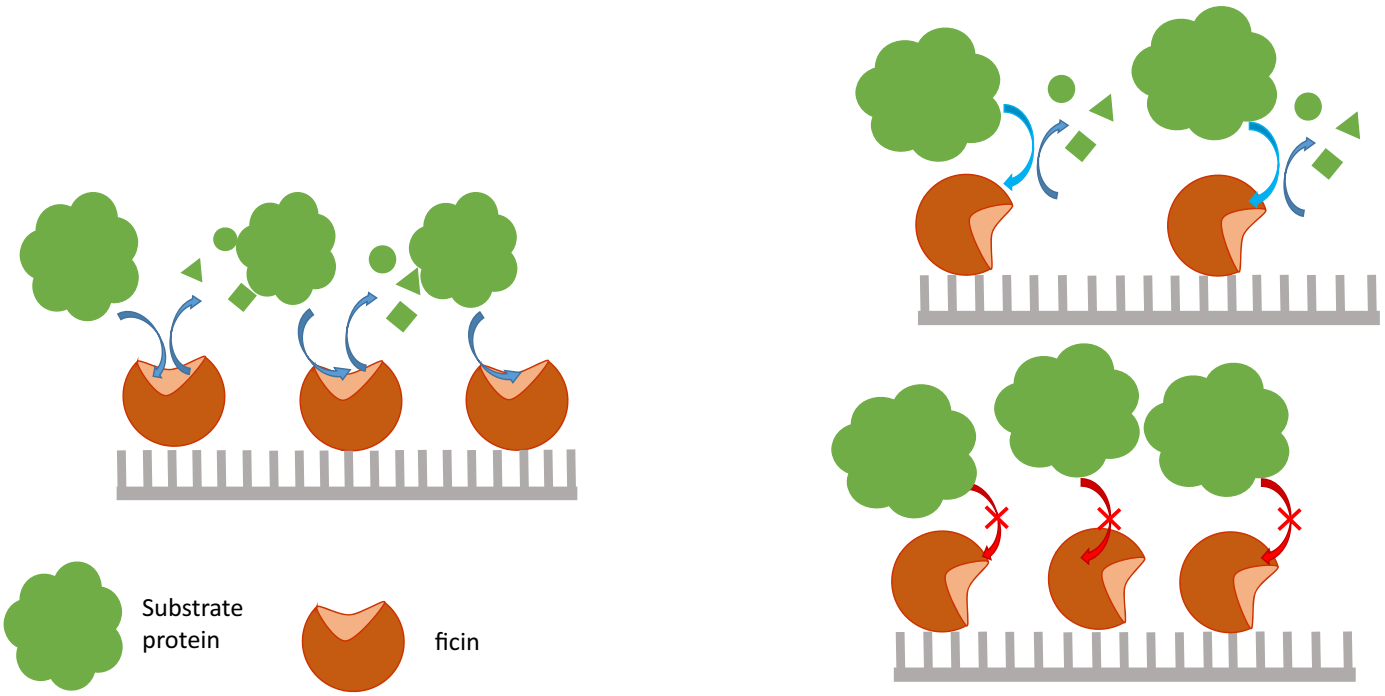

Fig. 2. Effect of the enzyme loading on the activity of the enzyme against a large protein: importance of the enzyme orientation.

benzoyl-D,L-arginine p-nitroanilide hydrochloride and 90% versus casein [77]. Aminated ficin was immobilized on glyoxyl agarose beads, and after optimization the activity versus benzoyl-D,L-arginine p-nitroanilide hydrochloride was lower than that of the immobilized and non-aminated enzyme, but it was higher using casein as substrate. The new biocatalyst was more stable than the reference mainly at pH 7. This catalyst was more active than the free enzyme or the immobilized and not aminated in 8 M urea, at pH 7 and 55 °C. Very interestingly, the aminated enzyme maintained high proteolytic activity when fully loading the support with enzyme, while using the non-aminated enzyme the activity versus casein drastically dropped using a fully loaded biocatalyst, [77]. Following the same strategy, in an attempt to improve the enzyme stability by crosslinking with glutaraldehyde was carried out. The effects on enzyme stability modifying the immobilized enzyme with glutaraldehyde were quite significant, improving enzyme activity and stability in certain circumstances [76]. When the immobilized enzyme was aminated, an increase in enzyme activity versus casein and a decrease using the ester was detected. The immobilized and aminated enzyme was more stable at pH 5 and less stable at pH 9 than the non-aminated enzyme. When the researchers tried to couple both modifications to get a more intense crosslinking, the enzyme activity was almost fully lost, making this unsuitable, even though results in aminated enzyme immobilization had been very positive (see above) [76].

Finally, ficin has been immobilized on glutaraldehyde activated agarose and used as a model enzyme to show the versatility of this method [78]. Immobilization via ion exchange on aminated agarose was very poor, and that prevented trying the strategy of glutaraldehyde treatment of previously ionically exchanged ficin [79]. Ficin could be properly immobilized on an aminated support preactivated with glutaraldehyde at pH 5 and better at pH 7, while at pH 9 the enzyme was almost fully inactivated. At pH 7, immobilization yield was 100% with an expressed activity of 40% versus benzoyl-D,L-arginine p-nitroanilide hydrochloride and 30% versus casein. The immobilization effects on enzyme stability depended on the substrate utilized to follow the activity and on the inactivation [79].

Ficin immobilized on glyoxyl agarose beads has been utilized in milk aggregation [80]. The results show that in the coagulation step the main point is the load of ficin in the support and not the total amount of ficin. In fact using lowly loaded biocatalysts casein aggregation was not observed even using a large excess of ficin, while the coagulation activity was very high using highly loaded enzyme preparations, although this presented a lower caseinolytic activity. Performing the hydrolysis with the highly loaded biocatalysts of casein in milk at low temperature to prevent coagulation [74,75,81–90] and later heating the hydrolysate, a high yield in a compact coagulum could be obtained [80].

Next, we will present some of the applications of ficin, in some instances the use of immobilized enzymes is not possible, while in other cases the use of the immobilized biocatalysts can open new opportunities.

3. Ficin applications as a protease

In some instances, ficin is applied just as a protease to check if the responsible of some property described in a crude extract is a protein (e.g., to check if after ficin treatment this extract property remains or disappears) or to check the stability of some antibodies [91–100]. However, we will focus in more specific applications of the enzyme as industrial catalysts (Fig. 3).

3.1. Proteolysis of proteins

This is the most straightforward application of a protease: to hydrolyze proteins. Collagen has been extracted from cattle tendons after pretreatment with ficin and pepsin, with a significantly lower amount of ficin being required to get optimal results [101]. Canola protein has been proteolyzed using trypsin, ficin and/or bromelin, combining this treatment with transglutaminase. The effects of these treatments on the gelation of the protein were analyzed [102]. Proteolysis produced a decrease in the gel strength, but a limited proteolysis permitted a better crosslinking using transglutaminase [103,104] producing a stronger

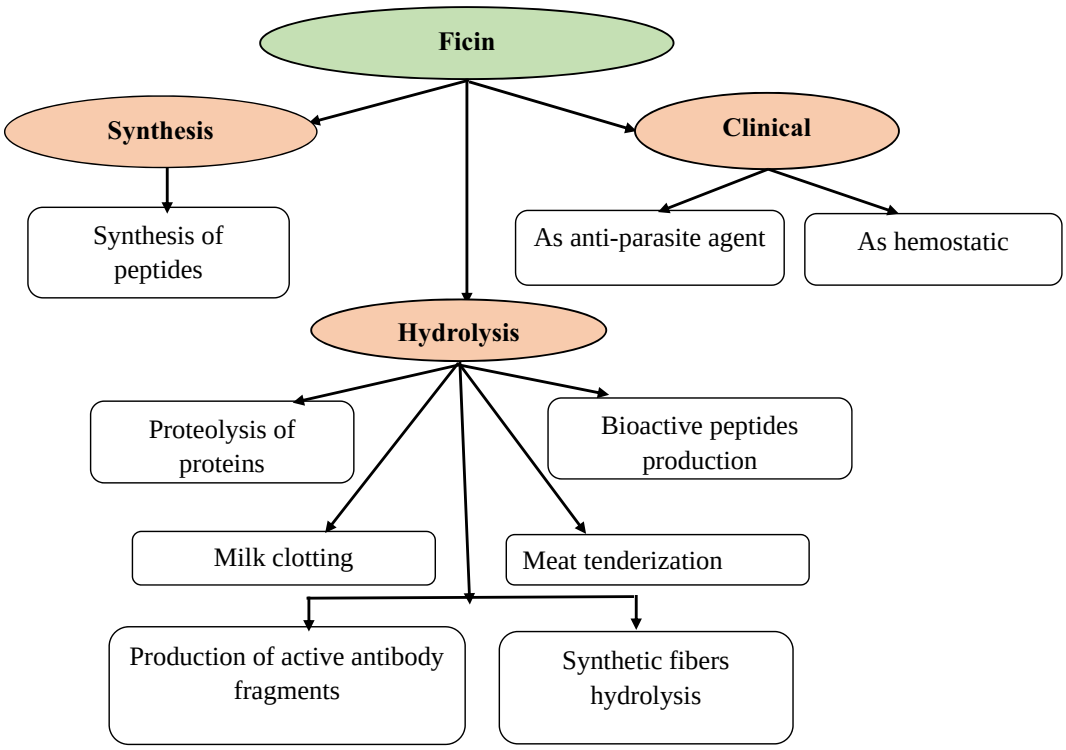

Fig. 3. Different applications of ficin.

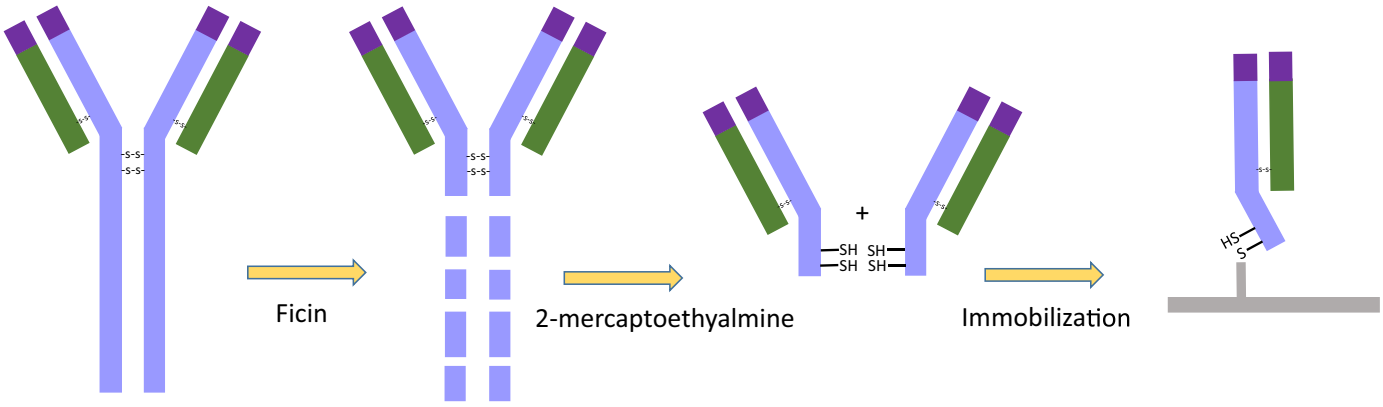

Fig. 4. Use of ficin to produce active antibody fragments and their immobilization on thiol reactive surfaces.

gel. The foaming capacity of hordein from barley has been increased by limited hydrolysis with ficin and papain, although excessive proteolysis produces a decrease in the foam stability. [105]. However, they cannot hydrolyze albumin.

Hydrolysis of soybean meal, fish and barley proteins catalyzed by ficin, papain and protease from Streptomyces griseus was utilized to predict ruminal protein degradability [106]. Soybean meal nitrogen was almost completely hydrolyzed; whereas barley proteins in vitro hydrolysis was slow to moderate (this was explained by a poor accessibility of structural proteins to the proteases) [106].

3.2. Production of active antibodies fragments by proteolysis

Certain antibodies applications may be improved if using only the active fragment antigen-binding (Fab) units of the protein [107,108] (Fig. 4). For example, the F(c) portion of IgG molecules are used to analyze the biological effect-binding to the F(c) receptor, mediating antibody-dependent cellular cytotoxicity, and complement fixation [109]. Moreover, it has been reported that IgMs fragments bear a resemblance to IgG in structure and size but they may have a lower binding affinity [110]. The Fc portion of IgM can function as complement activation making the production of fragments of IgM for both cytotoxicity studies and for in vivo use are desirable.

Thus, proteases have been used to produce these fragments. For example, five different mouse monoclonal antibodies were submitted to digestion catalyzed by ficin, and also elastase, bromelain and pepsin were utilized. The objective was to get active F(ab)2 fragments [111]. Elastase gave no digestion, while pepsin gave reduction of IgG to small inactive fragments while it was unable to digest some of the immunoglobulins, immune-activity of the antibodies fragments was not always preserved. Bromelain and ficin gave excellent results, giving in all cases a rapid and reproducible response for all assayed antibodies, the five antibodies being reduced to active F(ab)2. The authors state that ficin-obtained F(ab)2 exhibited a highly conserved immunoreactivity [111].

A specific anti-epidermal growth factor receptor (antiEGFRvIII) is used in the diagnostics of several tumors [69]. The use of the whole antibody raises some problems, like long-term circulation, retention and enhanced retention and permeability effects. This has been solved by using the Fab fragment of 4G1 (Fab-4G1), obtained by hydrolysis of the whole antibody with immobilized ficin and then purified through a protein A column to generate the Fab fragment [69]. Similarly, immobilized ficin was used to digest glypican-3-antibody (a cell surface receptor), creating αGPC3-F(ab')2 fragments subsequently conjugated to 89Zr [112]. This permitted a F(ab')2-dependent, antigen-specific cell binding.

The proteolysis may alter the purification of the active antibodies fragments. For example, Ficin has been used to hydrolyze IgG, producing the protein cleavage at the hinge region [96]. To purify this, it has been recommended to use protein G as the fragments are poorly recognized by protein A.

In another application of ficin hydrolyzing antibodies, the selective biotinylation of antibodies at the hinge region has been reported [113]. First, the antibodies have been digested with ficin and later, the obtained F(ab')2 fragments have been incubated with activated biotin, permitting the site-directed immobilization on avidin supports.

3.3. Milk clotting

The potential of ficin in milk coagulation was reported a long time ago [114,115]. For example, ultrafiltered bovine skimmed milk samples were treated with calf rennet, ficin and papain [116]. The authors found that ficin and papain had a more significant effect on proteolysis in curd formed from regular and 1 × ultrafiltered milk than on 2× or 4 × ultrafiltered milk. The authors proposed that the ultrafiltration may produce structural changes to some milk constituents that alter the clotting properties and/or proteolysis of the casein molecules [116].

In another research, ficin was compared to Polyporus badius extract in the coagulation of milk by analyzing the rheological and microstructural characteristics of ewes milk curd [117]. The gels produced by P. badius gels were more viscous and with a softer texture than the ficin gels. That is, they have higher moisture and lower protein contents presenting a more compact structure [117]. In another example, the coagulation of ewe milk was produced using extracts from Cynara scolymus L. cv. Blanca and ficin, comparing their performance to that of chymosin [118]. The coagulum obtained with ficin from the latex of Ficus carica had a higher yield (due to the high water retention capacity).

3.4. Bioactive peptides production

The production of bioactive peptides is one of the areas of utmost interest in the use of proteases. For instance, gelatin hydrolysate from Uroteuthis duvauceli (an Indian squid) was produced using ficin [119]. The hydrolysate properties were evaluated as anti-breast cancer agents (matrix-metalloproteinases zymography, cell-migration, apoptosis/necrosis, phase-contrast morphological examination, cytotoxicity and clonal-growth). MCF-7 and MDA-MB-231 breast cancer cells were used as problem samples, while HUVEC normal cells were used as reference. Significant inhibition of MCF-7 and MDA-MB-231 with no cytotoxicity on HUVEC cells was detected. In living mice, gelatin hydrolysate induced p53, avoided weight loss, reduced levels of Ki67 and diminished tumor size. [119].

In another example, wheat gliadins were hydrolyzed using ficin, and also with elastase, chymotrypsin, and pepsin [120]. The produced

peptides presented inhibition potential of dipeptidyl peptidase and they also presented antihypertensive and antioxidant features.

In another research, peptides were produced from goat milk casein by proteolysis catalyzed by trypsin and/or ficin [121]. The hydrolysate antimicrobial activity against both Gram-positive and Gram-negative bacteria was analyzed [121]. Hydrolysis increased the antimicrobial activity of the goat milk casein, being the most active the sample obtained using ficin. One peptide presented the highest activity against Escherichia coli and Bacillus cereus [121].

Bovine casein was hydrolyzed using ficin, and the produced hydrolysate was evaluated as antioxidant product [122]. Eight peptides were identified with potential antioxidant properties [122]. Another example shows that bovine hepatic extract was prepared by enzymatic hydrolysis using different enzymes (bromelain, ficin, pancreatin, and protease NP) but the best detoxifying activity was obtained using papain [123].

Ficin was used together with other proteases to hydrolyze a cellulase-treated defatted flaxseed meal, isolating the low molecular weight and the cationic peptide fractions [124]. All peptides presented antioxidant activities (nitric oxide, electron-spin resonance-detected hydroxyl radical, superoxide anion radical and 2,2-diphenyl-1-picrylhydrazyl radical, and inhibiting semicarbazide-sensitive amine oxidase activity). The low molecular weight fractions produced using ficin (and also using pepsin and papain) may also act as anti-inflammatory agents [124].

3.5. Meat tenderization

Ficin has been used for meat tenderization for a long time [125,126]. This ficin use has been recently reviewed [127]. For example, Triceps brachii and Supraspinatus were submitted to treatment with ficin, protease from Bacillus subtilis, homogenized fresh ginger, bromelain, papain, and two proteases from Aspergillus oryzae [128]. Control samples presented less water soluble proteins that ficin (which was the exception). All enzyme treatments increased meat tenderness via collagenous and myofibrin degradation, and the treatments did not present differences among high and low-connective tissue muscles [128].

In another paper, the meat-tenderizing properties of ficin, calpain, bromelain and papain were compared using lean beef strips, following the changes in the meat by IR spectra analyzed by chemometric techniques [129]. While ficin was the enzyme promoting the largest changes in factor 1 scores, the other enzymes were more efficient in factor 2. Eigenvalues calculated from IR spectra of ficin -and calpain- treated beef increased monotonically with time [129]. In another communication, the use of 50 or 100 ppm at 4 °C of ficin, papain or bromelain was compared to tender camel meat, studying quality textural changes, attributes and protein degradation, being papain the enzyme presenting the highest effect [130].

Also, ovine and bovine meats were treated with ficin to study the solubilization of meat proteins [131]. The increase of enzyme activity or time increased protein solubility, but if a very high activity was used, a massive breakage of myofibrillar produced very small peptides [131]. Furthermore, proteolysis of mechanically separated meat using ficin, bromelain and papain permitted the digestion of soft tissue without affecting the total bone fragments [132].

Bologna was produced using cysteine-modified soy proteins and ficin-tenderized meat [133]. Both treatments substantially improved water-holding capacity, emulsion stability, texture, and protein solubility.

3.6. Use of *fi*cin to catalyze amidation reactions

The use of proteases in peptide synthesis from aminoacids is one of the uses of these enzymes [5], and ficin is not an exception. N-Boc-Ala-OpGu and Ala-pNA were used as substrates using ficin as catalyst [134] to produce N-Boc-Ala-Ala.. Later on, 10 different amino acids were employed, obtaining 72–96% yields (depending on the amino acid) in a very rapid fashion [134].

In another paper, N-protected l-aminoacyl- and l-peptidyl-antipyrine amides were synthetized using different proteases, including ficin [135]. Optimization, using precipitation of the product, permitted to reach 100%.

Ficin, bromelain and papain were utilized as catalysts to produce Z-L-aminoacyl-L-caprolactam amides from Z-protected amino acid esters and DL-a-amino-e-caprolactam using a kinetically controlled strategy [136–138]. The obtained maximum yields were 96% and 87% for Z-Gly-L-caprolactam and Z-Ala-L-caprolactam, respectively [139]. Ficin has also been used to produce cationic heterooligopeptides (between 7 and 10 amino acids) using a kinetically controlled strategy [137,138], utilizing LysOEt and MetOEt as substrates, with a 49.5% (w/w) yield.

The kinetically controlled synthesis [137,138] of N-benzyloxycarbonyl (Z)-dipeptides was carried out by the use of free amino acids as nucleophiles, N-protected amino acid carbamoylmethyl esters as activated acyl donor and papain or ficin as catalysts, with similar results using both enzymes [140].

3.7. Use of *fi*cin on synthetic *fi*bers hydrolysis

This may have interest in biodegradation or in textile ageing. Lysine diisocyanate based polyurethanes were efficiently hydrolyzed by ficin, bromelain, and papain, showing high activity with this substrate [141]. Copoly(N-hydroxypropyl-(L)-glutamine/(L)-alanine) fibers were hydrolyzed using ficin, with results indicating that the degradation of the fibers occurred gradually from their surface into their core [142]. These treatments produced weaker fibers.

3.8. Clinical uses of *fi*cin

3.8.1. Use of *fi*cin as anti-parasite agent

Nowadays, resistance of intestinal parasitic helminth to standard drugs calls for the development of new anthelmintics (for a review on the matter see [143]). Fitting with their likely natural function [29,30] cysteine proteinases (including ficin) from fruits and protective plant lattices have been proposed as novel anthelmintics [144]. One study compared the in vitro anthelmintic effectiveness of cysteine proteinases from kiwi fruit, papaya, fig, pineapple and Egyptian milkweed versus Heligmosomoides polygyrus [145]. Except kiwi fruit extract, all proteases damaged the cuticle of adult H. polygyrus worms. Efficacy depended on the presence of cysteine, being ficin one of the most effective. They have been proved to be also effective versus Heligmosomoides polygyrus, Protospirura muricola and Trichuris muris (rodent nematodes) [146].

3.8.2. Use of *fi*cin as hemostatic

Ficin from Ficus carica presents hemostatic potency. This was explained because it reduces the prothrombin time of normal plasmas and plasmas deficient in coagulation factors and the activated partial thromboplastin time [147]. Two of the ficin components behave as factor X activators (via successive hydrolysis in the heavy chain between Leu178 and Asp179, Arg187 and Gly188, and Arg194 and Ile195 and the release of a carboxy-terminal peptide). The cleavage pattern of FXa degradation products in the light chain was influenced by Ca^{2+} and Mn^{2+}. These data suggest the hemostatic potential of Ficus proteases is based on the activation of the human coagulation factor X [147]. In another research, the use of a recombinant two-component composite formed by recombinant prothrombin (rfII) and activated factor X (rfXa) has proved to permit a linear dose-dependent increase in the rate of thrombin generation [148].

3.8.3. Other clinical uses of *fi*cin

Monoclonal antibodies (e.g., TS1 versus cytokeratin 8) are used to locate drugs in tumor therapy, but these antibodies need to be eliminated

from the circulation to prevent likely negative side-effects. Several derivatives of the antiidiotypic antibody αTS1, intact monoclonal αTS1, scFv of a αTS1 and αTS1 Fab and Fab'2 fragments were produced by recombinant technology or by cleavage with Ficin to study the clearing capacity [149]. The whole divalent antiidiotypic IgG was the most efficient, the fragments showing a lower stability.

4. Ficin promiscuous activities: enlarging the range of ficin application out of proteases range

Ficin has been described to display a promiscuous activity in catalyzing the direct asymmetric aldol reactions of different heterocyclic ketones (containing nitrogen, sulfur or oxygen) with aromatic aldehydes [150]. Some authors have found some promiscuous peroxidase activity related to ficin. Some proofs of the intrinsic peroxidase-like activity of ficin have been supplied [151]. This way, the enzyme can transform peroxidase substrates to colored products in the presence of hydrogen peroxide, being the active site of the protease activity different to that of the peroxidase activity. Ficin peroxidase activity was utilized to in situ synthesize intrinsic fluorescent polydopamine nanoparticles and to develop a rapid dopamine sensing method [152]. This was based on the ficin-peroxidase oxidization of dopamine to its quinone derivative and the subsequent autopolymerization of this compound into fluorescent polydopamine nanoparticles in the presence of H_2O_2. Using ficin as a peroxidase, the reaction of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic) acid and H_2O_2 was studied [153]. L-Cys was a competitive substrate, which resulted in fading of the chromogenic reaction, and permitted to determinate the concentration of the aminoacid. In another paper, the peroxidase activity of ficin was improved (between 1,7 and 3 folds) binding a heme group to the protease [154]. This modified enzyme was used for the successful determination of uric acid in human serum.

5. Conclusion and future trends

Ficin is an example of the application of an enzyme before really knowing the mechanism of action and all its physical properties. Perhaps this is due to the diversity of available ficin extracts (depending on the Ficus species), the changes in the ficin components over the season or even the environmental conditions, that makes characterizing an enzyme cocktail under always changing conditions challenging. The growing interest of academy in ficin is due to the new applications that this proteolytic extract is finding (e.g, active antibiotic fragments production, promiscuous activity) and also to the higher attention that some classical applications are raising, like milk clotting or meat tenderization. This last application is due to the fact that vegetable proteases are more compatible with health perception of the consumers than animal or bacterial recombinant proteases, mainly after the creasy-cows illness episode. Ficin has been commercially available from Sigma for a long time (perhaps at a relatively high price, around 1 euro per unit) or may be directly obtained from fig sap, although this may be subject to some irreproducibility on the samples composition, and thereforel their features may be not fully reproducible. As it is present in many species of the Ficus genus, the variety of enzyme functionalities (stability, range of pH, etc) becomes huge and their likely applications may increase. The use of Ficus cell cultures may be a good solution to get a reasonably cheap product with similar composition comparing different batches. The fact that ficin extract is really a cocktail formed by diverse enzymes may be an advantage for some of the applications, as it has been recently reviewed in a new biocatalysts concept called combi-enzymes [155] mainly for modification of multifunctional substrates, but also with interest in simpler processes.

On the other hand, the efforts to get properly immobilized ficin biocatalyst have offered good results in different areas, as surprising good performance of highly loaded ficin immobilized biocatalysts in glyoxyl agarose to milk clotting [80]. Although these results may open new opportunities to the use of the ficin, the use of nanoparticles as supports for ficin immobilization may have special interest for some applications, considering that some substrates are not soluble and that make the use of porous supports not possible [156].

Another factor that can delay the implementation of this protease extract, may be the lack of proper analysis of the specificity of all components of the ficin extract. The scarce data suggest a quite unspecific protease with a certain preference by aromatic amino acids, but deeper studies in this matter are required. However, even with these problems, ficin uses at academic level are experimenting a growing interest, considering the increasing number of publications that contains the term ficin in abstract or key words (12 papers in 2000, 28 in 2019 following Scopus). Very likely, the number of papers and industrial applications of ficin will increase in the next future.

Author statement

This is review paper, RFL designed the structure and supervised the writing, editing the final version, ABM edited the final version, RMS and HES performed the bibliographic search and write the preliminary draft, OLT write the general introduction and help in the final editing of the review.

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Conclusion and future trends

Ficin is an example of the application of an enzyme before really knowing the mechanism of action and all its physical properties. Perhaps this is due to the diversity of available ficin extracts (depending on the Ficus species), the changes in the ficin components over the season or even the environmental conditions, that makes characterizing an enzyme cocktail under always changing conditions challenging. The growing interest of academy in ficin is due to the new applications that this proteolytic extract is finding (e.g, active antibiotic fragments production, promiscuous activity) and also to the higher attention that some classical applications are raising, likemilk clotting ormeat tenderization.

This last application is due to the fact that vegetable proteases are more compatible with health perception of the consumers than animal or bacterial recombinant proteases, mainly after the creasy-cows illness episode. Ficin has been commercially available from Sigma for a long time (perhaps at a relatively high price, around 1 euro per unit) or may be directly obtained from fig sap, although this may be subject to some irreproducibility on the samples composition, and therefore their features may be not fully reproducible. As it is present in many species of the Ficus genus, the variety of enzyme functionalities (stability, range of pH, etc) becomes huge and their likely applications may increase. The use of Ficus cell cultures may be a good solution to get a reasonably cheap productwith similar composition comparing different batches. The fact that ficin extract is really a cocktail formed by diverse enzymes may be an advantage for some of the applications, as it has been recently reviewed in a new biocatalysts concept called combienzymes [155] mainly for modification of multifunctional substrates, but also with interest in simpler processes.

On the other hand, the efforts to get properly immobilized ficin biocatalyst have offered good results in different areas, as surprising good performance of highly loaded ficin immobilized biocatalysts in glyoxyl agarose to milk clotting [80]. Although these results may open new opportunities to the use of the ficin, the use of nanoparticles as supports for ficin immobilization may have special interest for some applications, considering that some substrates are not soluble and that make the use of porous supports not possible [156]. Another factor that can delay the implementation of this protease extract, may be the lack of proper analysis of the specificity of all components of the ficin extract. The scarce data suggest a quite unspecific protease with a certain preference by aromatic amino acids, but deeper studies in this matter are required. However, even with these problems, ficin uses at academic level are experimenting a growing interest, considering

the increasing number of publications that contains the term ficin in abstract or key words (12 papers in 2000, 28 in 2019 following Scopus). Very likely, the number of papers and industrial applications of ficin will increase in the next future.

Conclusion et perspectives

La ficine est un exemple d'application d'une enzyme avant de connaître son mécanisme d'action et toutes ses propriétés physiques. Cela est peut-être dû à la diversité des extraits de ficine disponibles (selon les espèces de Ficus), aux changements des composants de la ficine au cours de la saison ou même aux conditions environnementales, ce qui rend difficile la caractérisation d'un cocktail enzymatique dans des conditions toujours changeantes. L'intérêt croissant de la recherche pour la ficine est dû aux nouvelles applications que cet extrait protéolytique trouve (par exemple, production de fragments d'antibiotiques actifs) et également à l'attention accrue que suscitent certaines applications classiques, comme la coagulation du lait ou l'attendrissement de la viande.

Cette dernière application est due au fait que les protéases végétales sont plus compatibles avec la perception sanitaire des consommateurs que les protéases animales ou bactériennes, principalement après l'épisode de maladie des vaches folle. La ficine est disponible dans le commerce auprès de Sigma depuis longtemps (peut-être à un prix relativement élevé, environ 1 euro par unité) ou peut être directement obtenue à partir de latex de figue, bien que cela puisse être sujet à une certaine irreproductibilité sur la composition des échantillons, et donc leur fonctions peuvent ne pas être entièrement reproductibles. Comme il est présent dans de nombreuses espèces du genre Ficus, la variété des fonctionnalités enzymatiques (stabilité, plage de pH, etc.) devient énorme et leurs applications probables peuvent augmenter. L'utilisation de cultures de cellules de Ficus peut être une bonne solution pour obtenir un produit raisonnablement bon marché avec une composition similaire comparant différents lots. Le fait que l'extrait de ficine soit vraiment un cocktail formé par diverses enzymes peut être un avantage pour certaines applications, car il a été récemment montré dans un nouveau biocatalyseur appelé combi-enzymes [155] principalement pour la modification de substrats multifonctionnels, mais aussi avec intérêt dans des processus plus simples.

D'autre part, les efforts pour obtenir un biocatalyseur de ficine correctement immobilisé ont offert de bons résultats dans différents domaines, comme les performances surprenantes de biocatalyseurs de ficine immobilisés dans le glyoxyl-agarose hautement chargés pour la coagulation du lait [80]. Bien que ces résultats puissent ouvrir de nouvelles opportunités à l'utilisation de la ficine, l'utilisation de nanoparticules comme supports d'immobilisation de la ficine peut présenter un intérêt particulier pour certaines applications, étant donné que certains substrats ne sont pas solubles et que l'utilisation de supports poreux n'est pas possible [156].

Un autre facteur qui peut retarder la mise en œuvre de cet extrait de protéase peut être le manque d'analyse appropriée de la spécificité de tous les composants de l'extrait de ficine. Les rares données suggèrent une protéase non spécifique avec une certaine préférence pour les acides aminés aromatiques, mais des études plus approfondies dans ce domaine sont nécessaires. Cependant, même avec ces problèmes, les utilisations de la ficine au niveau expérimental suscitent un intérêt croissant, compte tenu d e nombre croissant de publications contenant le terme ficine en résumé ou en mots clés (12 articles en 2000, 28 en 2019 après Scopus). Très probablement, le nombre de papiers et d'applications industrielles de la ficine augmentera dans le prochain avenir.

EXPEREMENTAL PART

MATERIAL AND METHODES

1. Fig latex collect and ficin extraction

Fresh fig latex was collected by breaking the immature green fruits and leaves of *Ficus carica L*. growing in Kabylia, a Mediterranean region (Tizi ougueni, Adekar, Bejaia, Algeria), and collecting the fluid in a clean flask at 4 °C. All the latex samples used in this study were collected freshly from the fig tree in the summer (June and jully 2016, 2017). The latex was stored in the refrigerator (4 to 8°C) until the enzyme system is extracted.

To extract the enzymes the latex was centrifuged at 3200 g for 15 min at 4 °C (Nouani *et al.*, 2009) to remove gums and other debris. The insoluble material was discarded and the supernatant was retained and represented the "crude extract of ficin", stored at −20 °C until use.

The protein concentration was quantified by Bradford's method (Bradfort, 1976). BSA was used as the standard protein.

2. SDS-PAGE analysis of the ficin extract

SDS–PAGE was carried out according to LAEMMELI (1970) using 12% separating and 4% stacking gels.

The samples were mixed at a ratio of 1 mg of protein per mL of the buffer sample at pH 6.8 (0.378 g TRIS base, 1 g of SDS, 2.5 mL of β-mercaptoethanol and 5 mL of glycerin. The volume was adjusted with H2O until 25 mL).

Twenty micrograms of proteins were loaded on the gel and then subjected to separation in a running buffer containing 25 mM of Tris, 192 mM glycine and 0,1% SDS at a pH of 8,3 for 2 h at 100 V.

After separation, the gel was stained for 1 h with a staining solution containing 3 mM Coomassie Brilliant Blue R-250 in 40% (v/v) ethanol, and 10% (v/v) acetic acid. Protein patterns were visualized after de-staining the gel in 40% (v/v) ethanol, and 10% (v/v) acetic acid until a clear background was achieved. A mixture of protein molecular weight markers obtained from Bio-Rad was used.

3. Enzymatic Assays

Ficin activity was determined by two different protocols, using small synthetic substrate benzoyl-D, L-arginine p-nitroanilide hydrochloride (BANA) or a large natural substrate casein.

BANA was used following the method described by Devaraj *et al.*, (2008). 43.5 mg of BANA were dissolved in 1.0 mL of dimethyl sulfoxide. Then, it was diluted to a total volume of 100 mL in 0.1 M sodium phosphate pH 7, containing 5mMEDTA and 5mM cysteine. The enzyme activity was determined by measuring the absorbance of the pnitroaniline formed in this hydrolytic reaction at 405 nm (ε was 8800 for p-nitroaniline under these conditions). One unit of BANA activity was expressed as the amount of p-nitroaniline formed per 1 mg of enzyme per min.

Casein was used following themethod described by Kunitz (1947) with slight modifications. A solution of 1% (w/v) casein was prepared in 100 mM sodium phosphate at pH 7.0 containing 5 mM cysteine and 5 mM EDTA at 55 °C. To 1 mL of substrate solution, 200 μL 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 2 mL of 10% trichloroacetic acid (TCA), then it was incubated for 10 min at room temperature and centrifuged at 10,000 rpm. This treatment produces the precipitation of the protein, but the small peptides are maintained in solution. The absorbance of 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 casein activity is defined as the amount of enzyme that increases the absorbance by 0.001 min−1 under the given assay conditions.

4. Preparation of supports (Agarose Beads)

4.1. Glyoxyl agarose

Glyoxyl agarose was prepared as previously described (MATEO *et al.,* 2005 and GRAZU *et al.,* 2006).

A- The first step is Activation of Agarose Gel to Glyceryl-Agarose.

- 52,5 g of agarose 4B in 128,5 mL of distilled water. Then add 35,75 ml of 1.7 N NaOH containing 2.43 g sodium borohydride.

- This suspension was maintained in an ice bucket under mechanical stirring, and glycidol was added drop wise in order to reach a 2 M final concentration (Glycidol addition must be very slowly to prevent the temperature rising over 25 °C).

- Keep the suspension under mild stirring overnight (18 h) at 25 °C.

- Filter and wash the support thoroughly with distilled water.

B- The seconds step is Oxidation of Glyceryl-Agarose to Glyoxyl-Agarose (Oxidation of glycols with sodium periodate is a stoichiometric reaction. Therefore, the activation degree of the support can be easily controlled through the periodate concentration used)

- The sodium periodate was added slowly (80 µ mol of NaIO4 per wet gram of beads to the activated gel or 10 mL of the suspension). This oxidative reaction was allowed to proceed for 3 h under mild stirring at room temperature. Then, the glyoxyl support was washed with an excess of distilled water and stored at 4 °C under wet conditions.

4.2. MANAE- supports

MANAE was prepared from glyoxyl supports with a modification of the protocol previously described (MATEO, *et al.*, 2006 and FERNÁNDEZ-LAFUENTE, *et al.*, 1993) ethylenediamine/glyoxyl agarose beads reaction time was 24 h before reduction.

Suspend 12,5g of glyoxyl aggarose in 50 mL of 2M ethileneamine pH 10 this suspension was maintained under mild stirring for (24H). After that 10mg/mL (or 10 mg/g support) of sodium borohydrid was added and the suspension was stirred for 24 hours. then the support was washed with the sodium carbonate 150 mM at pH 9 and sodium acetate 100 mM at pH 5 (approximatively 1L for each buffer) then with excess of distilled water. MANAE was stored at 4ºC.

4.3. Preparation of Glutaraldehyde Agarose Beads

Activation of MANAE to glutaraldehyde agarose was prepared as described by BARBOSA, *et al.*, 2012, CORMA, *et al.*, 2001 and BETANCOR, *et al.*, 2006. (This protocol guarantees that each primary amino in the support has been modified with two glutaraldehyde molecules).

- Prepare 10% glutaraldehyde solution in 200 mM sodium phosphate buffer and adjust the pH at 7.0 (pH should be controlled as glutaraldehyde may polymerize under basic pHs, decreasing the degree of functionalization and therefore preventing enzyme immobilization by covalent interaction).
- Suspend 50 grams of MANAE agarose beads was suspended in 100 glutaraldehyde solution prepared.
- Keep the suspension under mild stirring at 25 °C for 24 h (the suspension color is an indicator of the polymerization degree. If the suspension is brownish colored the support cannot be used since glutaraldehyde polymerization has occurred). Filter and wash exhaustively the suspension with 25 mM sodium phosphate buffer pH 7.0 (5 volumes) and then with an excess of distilled water (the glutaraldehyde activated agarose must be used immediately after filtration).

5. Immobilization of ficin extract

5.1.On glyoxyl-support

Ficin extract immobilization was performed by adding 10 g of glyoxyl agarose to 100 mL of ficin extract solution (protein concentration was 1 mg/mL) prepared in 50 mM sodium carbonate at pH 10.0 at room temperature under continuous stirring. A loading of 10 mg protein per g of support loading (maximum loading of this support is 30 mg/g) was selected to ensure a proper study of the effect of the immobilization in the enzyme properties without diffusion problems (RODRIGUES, *et al.*, 2013).

As a reaction end point, derivatives were reduced by addition of solid NaBH4 to reach a concentration of 1 mg/mL (MATEO, *et al.*, 2005). After gentle stirring for 30 min at room temperature, the resulting derivatives were washed with abundant distilled water to eliminate residual sodium borohydride. Immobilization yield is defined as the percentage of offered enzyme that become immobilized on the support, while expressed activity is defined as the ration between observed activities and theoretical immobilized activities.

Samples from suspensions and supernatants were periodically withdrawn and their catalytic activity determined using BANA and casein. Immobilization yield is defined as the percentage of offered enzyme that become immobilized on the support, while expressed activity is defined as the ration between observed activities and theoretical immobilized activities.

5.2. On MANAE-Agarose and glutaraldehyde-agarose

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 immobilization was performed at 25ºC (room temperature).

To show the maximum loading and the effect of the loading on the activity, 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.

6. Chemical modification of ficin

6.1. Studies of enzyme structure and aminoacid solvent accessibility

Protein structures were visualized using PyMol software version 0.99rc6 [DeLano W. Pymol: An open-source molecular graphics tool. CCP4 Newsl Protein Crystallogr 2002]. Solvent Accessibility Prediction of residues of 4YYQ, 4YYR, 4YYV and 4YYW were calculated by the web-based program PROFacc, PredictProtein server, (www. predictprotein.org). PROFacc predicts solvent accessibility of protein residues for 10 states of relative accessibility. These are grouped into two states: buried and exposed (ROST, *et al,*. 2004).

6.2. Chemical modification of the ficin extract immobilized on glyoxyl agarose beads

6.2.1. Amination of carboxylic groups

Amination of immobilized ficin was performed following the protocol described above (BARBOSA, *et al.,* 2012 and FERNANDEZ-LAFUENTE, *et al.,* 1995).

Eight grams of ficin immobilized on glyoxyl agarose beads was suspended in 80 mL solution of 1M ethylenediamine (EDA) at pH 4.75 or pH 6. Then, solid 1-Ethyl-3-(3-dimethylaminopropyl)-Carbodiimide (EDC) was added to the suspension to the desired concentration (1mM or 10 mM) or in 10mM EDC at pH 6. After 90 min of gentle stirring at 25 °C, 100% modification of the exposed carboxylic groups was achieved using 10mM EDC and pH 4.75, while 40–50% was modified using 1mM EDC or 10mM and pH 6.

In some instances, the modified ficin was filtered and 4 g were incubated for 24 h in a 0.1M hydroxylamine solution at pH 7 to recover the modified tyrosines (Caraway, *et al.*, 1968).

The final preparation was filtered and washed with 25mM sodium phosphate at pH 7.5 and with an excess of distilled water.

6.2.2. Modification with glutaraldehyde

A mass of 8g of glyoxyl-ficin were suspended in 80 ml of 25mM sodium phosphate at pH 7 containing 0.03%, 0.1% or 1% (v/v) of glutaraldehyde for 1 h. Then, the biocatalyst was washed with distilled water.

One gram was suspended in 10 ml of 25mM carbonate buffer pH 8.5 for 24 h. Activity was followed using the BANA assay during the modification.Then, 80 ml of water were added and sodium borohydride was added to reach a concentration of 1 mg/ ml, stirred for 30 min and washed with an excess of distilled water.

6.2.3. Amination of the free enzyme with EDC and EDA

The free ficin was added to 50 mL solution of 1 M EDA at pH 4.75. Solid EDC was added to the suspension to a final concentration of 10 mM. It has been reported that after 90 min of gentle stirring at 25 °C, 100%modification of all the exposed carboxylic groupswas achieved (HOARE, *et al.*, 1967).

The modified enzyme was then dialyzed at 4 °C for 24h using a dialysis membrane with a molecular weight cut off of 10 kD. Dialysis was carried out in 2.5L of 5 mM phosphate buffer, the dialysis buffer was changed after 2h, 4h, 10h and 24h.

In this part of work immobilization of aminated or non-aminated ficin on glyoxyl-agarose was performed as described previously bay SIAR *et al,*. (2017). In some cases, the enzyme immobilized at pH 9.0 was incubated after immobilization at pH 10.0.

To follow the immobilization courses, aliquots from suspensions and supernatants at the immobilization conditions maintained the activity unaltered during the whole immobilization process.

The maximum immobilization time was 3 h (optimal value found using the not aminated enzyme) (SIAR *et al,*. 2017). Then the derivatives were vacuum filtered and

washed thoroughly with distilled water. In some instances, the enzyme concentration was increased to increase the amount of offered enzyme (1, 3, 6 and 10 mg/mL were employed).

7. Thermal inactivation of ficin preparations

The different ficin preparations were incubated at 55℃ in 50mM sodium acetate at pH 5, 50 mM 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 assay described above. Stabilizations were calculated from the ratio between the half-lives obtained from the inactivation courses.

8. Use and re use of the Immobilized ficin in the hydrolysis of casein

The standard method of enzyme activity determination using casein was described by KUNITZ (1964) and here it has been used with some slight modifications (as described in section enzyme assay).

In some instances, in order to show the effect of Urea casein was prepared at the % as described but containing urea. Temperature was altered (from 55 to 70 °C), or urea to a concentration of 2 M was added.

In order to test the possibility of the reuse of the immobilized enzyme in the hydrolysis of casein, in the case of fcin-glyoxyl, five cycles of casein hydrolysis of 1 h were performed at 60 and 65 °C as described above. After 1 h of hydrolysis, the peptide production was checked and the immobilized enzyme was washed five times with 10 volumes of distilled water, and employed in a new reaction cycle.

For the ficin-glutaraldehyde six cycles of casein hydrolysis (2 h each) were performed at 50 ºC 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.

9. Use of glyoxyl-agarose immobilized ficin extract in milk coagulation

In this part of work immobilization of ficin was immobilized on glyoxyl-agarose. Different biocatalyst loadings were used (3, 10, 30 and 85mg/g) to show the effect of the loading on the milk clotting activity.

9.1.Casein clotting activity of ficin biocatalysts

The coagulant activity of immobilized and free ficin was determined following the method described by PESSELA *et al.* (2004) with slight modifications.

Casein from bovine milk was prepared at 1% (w/v) in 50 mM sodium acetate buffer containing 5 mM sodium chloride at pH 5.6. To start the reaction, the desired quantity of immobilized or free ficin was added to 2 mL of the substrate (casein solution 1%) at 25 °C and the reaction was followed spectrophotometrically by the increment of absorbance at 550 nm promoted by the protein precipitation. Activity was inversely proportional to the inflection point of this curve.

9.2.Two step casein coagulation

The hydrolysis of 1% (w/v) casein was first performed at 4 °C to prevent the precipitation of the hydrolysate (PESSELA, *et al.* 2004). Samples were withdrawn periodically, heated at 40 °C for 20 min to permit the precipitation of the hydrolyzed protein. Then, the absorbance was determined at 550 nm.

9.3.Two step clotting of skimmed milk

The first step of the coagulation of skimmed milk (Celta, pH 6), the enzymatic one, was carried out at 4 °C for 2.5 h (the volume of milk used was 40 mL using 40 mg of ficin in immobilized form or 300 μg of free ficin).

Then, when using immobilized enzymes, the samples were centrifuged to remove the biocatalyst taking advantage of the no precipitation of the casein hydrolysate at low temperature.

In a second phase, the supernatant was heated at 40 °C for 2 h (the time required to have a clear separation between coagulum and the whey) and coagulated casein was recovered by filtration (the filtration was carried out on a paper tissue for 15 min for each coagulum) and weighed. The yield was calculated according to the formula described by JEANTET, *et al.*, (2008).

Yield = (weight of recovered coagulum / weight of used milk) * 100.

9.4.SDS-PAGE analysis of the coagulum

SDS–PAGE was performed according to LAEMMELI (1970) using 12% separating and 4% stacking gels. The samples were mixed at a ratio of 1 mg of coagulum per mL of the buffer sample. The other steps were performed as described for the ficin extract.

1. Collecte de latex de figue et extraction de ficine

Le latex frais de figue a été recueilli en brisant les fruits verts immatures et les feuilles de *Ficus carica L* . poussant en Kabylie, région méditerranéenne (Tizi ougueni Adekar, Bejaia, Algérie), et récupérer le fluide dans un flacon propre à 4 ° C. Tous les échantillons de latex utilisés dans cette étude ont été prélevés fraîchement sur le figuier en été (juin et juillet 2016 , 2017). Le latex a été conservé au congélateur jusqu'à ce que le système enzymatique soit extrait.

Pour extraire les enzymes, le latex a été centrifugé à 3200 g pendant 15 min à 4 °C (Nouani *et al.*, 2009) pour éliminer les gommes et autres débris. Le culot a été jeté et le surnageant qui représente « l'extrait brut de ficine » a été récupéré, conservé à -20 ° C jusqu'à utilisation. La concentration en protéines a été déterminée par la méthode de Bradford (Bradfort , 1976). La BSA a été utilisée comme protéine standard.

2. Analyse SDS-PAGE de l'extrait de ficine

La SDS-PAGE a été réalisée selon LAEMMELI (1970) en utilisant 12% de gels de séparation et 4% d'empilage. Les échantillons ont été mélangés à un rapport de 1 mg de protéine par m L de l'échantillon tampon à pH 6,8 (0,378 g de base TRIS, 1 g de SDS, 2,5 mL de β - mercaptoéthanol et 5 mL de glycérine . Le volume a été ajusté avec H2O jusqu'à 25 ml) .

Vingt microgrammes de protéines ont été chargés sur le gel puis soumis à une séparation dans un tampon courant contenant 25 mM de Tris, 192 mM de glycine et 0,1% de SDS à un pH de 8,3 pendant 2 h à 100 V.

Après séparation, le gel a été coloré pendant 1 h avec une solution de coloration contenant 3 mM de Coomassie Brilliant Blue R-250 dans 40% (v / v) d'éthanol et 10% (v / v) d'acide acétique. Les motifs de protéines ont été visualisés après décoloration du gel dans de l'éthanol à 40% (v / v) et de l'acide acétique à 10% (v / v) jusqu'à l'obtention d'un fond clair . Un mélange de marqueurs de poids moléculaire protéiques obtenus auprès de Bio-Rad a été utilisé.

3. Détermination de l’activité enzymatique

L'activité de la ficine a été déterminée par deux protocoles différents, en utilisant un petit substrat synthétique benzoyl-D, L-arginine p-nitroanilide hydrochlorid (BANA) ou un gros substrat naturel de caséine .

La détermination de l'activité en utilisant le BANA selon la méthode décrite par Devaraj *et al.* , (2008) . 43,5 mg de BANA ont été dissous dans 1,0 mL de diméthylsulfoxyde. Ensuite, il a été dilué à un volume total de 100 ml dans du phosphate de sodium 0,1 M pH 7, contenant 5 mM EDTA et 5 mM de cystéine. L'activité enzymatique a été déterminée en mesurant l'absorbance de la pnitroaniline formée dans cette réaction hydrolytique à 405 nm (ε était de 8800 pour la p-nitroaniline dans ces conditions). Une unité d'activité BANA a été exprimée comme la quantité de p-nitroaniline formée pour 1 mg d'enzyme par minute.

La détermination de l'activité en utilisant la caséine a été réalisée selon la méthode décrite par Kunitz (1947) avec de légères modifications. Une solution de caséine à 1% (p / v) a été préparée dans du phosphate de sodium 100 mM à pH 7,0 contenant 5 mM de cysteine et 5 mM d' EDTA à 55 ° C. Pour 1 ml de solution de substrat, 200 µ l de la ficine (enzyme solution d'extrait ou immobilisée ficine suspension) a été ajouté, et le mélange réactionnel a été incubé à la température désirée pendant 20 min. La réaction a été arrêtée par l'addition de 2 ml d' acide trichloroacétique à 10% (TCA), puis elle a été incubée pendant 10 minutes à température ambiante et centrifugée à 10 000 tr / min. Ce traitement produit la précipitation de la protéine, mais les petits peptides sont maintenus en solution. L'absorbance du surnageant a été mesurée à 280 nm. Dans le cas de la référence, le substrat a été ajouté après inactivation de l'enzyme par incubation dans du TCA. Une unité d'activité de la caséine est définie comme la quantité d'enzyme qui augmente l'absorbance de 0,001 min-1 dans les conditions d'essai données.

4. Préparation des supports (billes d'agarose)

4.1. Glyoxyl agarose

Le glyoxyl agarose a été préparé comme décrit précédemment (MATEO *et al.*, 2005 et GRAZU *et al.*, 2006).

A- La première étape est l' activation du gel d'agarose en glycéryl-agarose .

- 52,5 g d'agarose 4B dans 128,5 mL d'eau distillée. Puis dd 35,75 ml de NaOH 1,7 N contenant 2,43 g de borohydrure de sodium.

- Cette suspension a été maintenue dans un seau de glace sous agitation mécanique, et on a ajouté du glycidol goute a goute jusqu'à parvenir à une concentration finale de 2 M (addition

glycidol doit être très lentement pour éviter l'élévation de la température au-dessus de 25 ° C) . Cette suspension est conservée sous agitation douce pendant une nuit (18 h) à 25 ° C.

- Filtrer et laver soigneusement le support avec de l'eau distillée.

B- Oxydation du glycéryl-agarose en glyoxyl -agarose

L'oxydation des glycols est obtenue avec les périodates de sodium. Le degré d'activation du support peut être facilement contrôlé grâce à la concentration de périodate utilisée)

- Le sodium de periodates a été ajouté lentement (80 µ mol de NaIO4 par gramme de support humides ou activé ou 10 ml de la suspension). On a laissé cette réaction d'oxydation se dérouler pendant 3 h sous agitation douce à température ambiante. Ensuite, le support glyoxyl a été lavé avec un excès d'eau distillée et stocké à 4 °C.

4.2. MANAE- prend en charge

Le MANAE a été préparé à partir de supports glyoxyl-agarose selon le protocole décrit précédemment avec des modification (MATEO , *et al.,* 2006 et FERNÁNDEZ - LAFUENTE , *et al.,* 1993) et le temps de réaction ethylènediamine / glyoxyl agarose était de 24 h..le protocole consiste a mettre en suspension 12,5 g de glyoxyl aggarose dans 50 ml d' éthylènediamine a une concentration de 2M et un pH 10 cette suspension a été maintenue sous agitation douce pendant (24H). Après cela, 10 mg / ml (ou 10 mg / g de support) de sodium borohydride ont été ajoutés et la suspension a été agitée pendant 24 heures. Puis, le support a été lavé avec du carbonate de sodium 150 mM à pH 9 et de l' acétate de sodium 100 mM à pH 5 (environ 1 L pour chaque tampon) puis avec un excès d' eau distillée .

4.3. Préparation de support actives avec le glutaraldéhyde

L'activation de MANAE en glutaraldéhyde agarose a été préparée comme décrit par BARBOSA , *et al.,* 2012, CORMA , *et al.,* 2001 et BETANCOR , *et al.,* 2006 . (Ce protocole garantit que chaque groupe amine primaire du support a été modifié avec deux molécules de glutaraldéhyde). Le protocole est comme suit :

- Préparer une solution de glutaraldéhyde à 10% dans un tampon phosphate de sodium 200 mM et ajuster le pH à 7,0 (le pH doit être contrôlé car le glutaraldéhyde peut polymériser à

des pH basiques, diminuant le degré de fonctionnalisation et empêchant ainsi l'immobilisation de l'enzyme par interaction covalente).

- Suspension de 50 grammes de MANAE-agarose dans 100 ml d'une solution de glutaraldéhyde.
- Maintenir la suspension sous agitation modérée à 25 ° C pendant 24 h (la couleur de la suspension est un indicateur de degré polymérisation. Si la suspension est brunâtre le support ne peuvent pas être utilisés étant donné que la polymérisation de glutaraldéhyde a eu lieu). Filtrer et laver complètement la suspension avec du tampon phosphate de sodium 25 mM pH 7,0 (5 volumes) puis avec un excès d'eau distillée (l'agarose activé au glutaraldéhyde doit être utilisé immédiatement après filtration).

5. Immobilisation d'extrait de ficine

5.1. Sur le glyoxyl -agarose

L'immobilisation de l'extrait de ficine a été réalisée en ajoutant 10 g de glyoxyl-agarose à 100 ml de solution d'extrait de ficine (la concentration en protéines était de 1 mg / ml) préparée dans du carbonate de sodium 50 mM à pH 10,0 à température ambiante sous agitation continue. Une charge de 10 mg de protéine par g de support (la charge maximale de ce support est de 30 mg / g) a été choisie pour assurer une bonne étude de l'effet de l'immobilisation sur les propriétés enzymatiques sans problèmes de diffusion (RODRIGUES , *et al.*, 2013) . En tant que étape final de la réaction, les dérivés ont été réduits par addition de NaBH4 solide jusqu'a une concentration de 1 mg/mL (MATEO , *et al.*, 2005). Après agitation douce pendant 30 min à température ambiante, les dérivés résultants ont été lavés avec de l'eau distillée pour éliminer le sodium de borohydide résiduel. Le rendement d'immobilisation est défini comme le pourcentage d'enzyme offerte qui s'immobilise sur le support, tandis que l'activité exprimée est définie comme le rapport entre les activités observées et les activités de l'enzyme après immobilisation. Des échantillons de suspensions et de surnageants ont été périodiquement prélevés et leur activité catalytique déterminée en utilisant du BANA et de la caséine .

5.2. Sur MANAE-Agarose et g glutaraldéhyde-agarose

Dix grammes (10 g) du support correspondant (MANAE-agarose ou glutaraldéhyde-agarose) ont été ajoutés à 100 mL d'extrait de ficine (contenant 1 mg de protéine / mL) dans de l'acétate de sodium 25 mM à pH 5, phosphate de sodium 25 mM à pH 6,5–8,5 ou 25 mM de carbonate de sodium à pH 9, dans tous les cas l'immobilisation a été réalisée à 25 °

C (température ambiante). Pour determiner la charge maximale et l'effet de la charge sur l'activité , la concentration d'enzyme a été augmentée pour augmenter la quantité d'enzyme offerte (un maximum de 12 mg / mL a été utilisé). Des échantillons de suspensions et de surnageants ont été périodiquement prélevés et leur activité catalytique déterminée en utilisant du BANA et de la caséine. Après 24 h, les biocatalyseurs ont été filtrés sous vide et lavés soigneusement avec de l'eau distillée.

6. Modification chimique de la ficine

6.1. Études de la structure enzymatique et de l'accessibilité aux solvants d'acides aminés

Les structures des protéines ont été visualisées à l'aide du logiciel PyMol version 0.99rc6 [DeLano W. Pymol : Un outil graphique moléculaire open-source. CCP4 Newsl Protein Crystallogr 2002]. Accessibilité aux solvants La prédiction des résidus de 4YYQ, 4YYR, 4YYV et 4YYW a été calculée par le programme Web PROFacc , serveur PredictProtein , (www. Predictprotein.org). PROFacc prédit l'accessibilité aux solvants des résidus protéiques pour 10 états d'accessibilité relative. Ceux-ci sont regroupés en deux états : enterrés et exposés (ROST, *et al,* 2004).

6.2. Modification chimique de l'extrait de ficine immobilisé sur billes de glyoxyl agarose

6.2.1. Amination des groupes carboxyliques

L'amination de la ficine immobilisée a été réalisée selon le protocole décrit ci-dessus (BARBOSA , *et al.,* 2012 et FERNANDEZ - LAFUENTE , *et al.,* 1995).

Huit grammes de ficine immobilisée sur glyoxyl agarose ont été mis en suspension dans une solution de 80 mL de 1 M d' éthylènediamine (EDA) , à pH 4,75 ou pH 6. Ensuite, l'1-éthyl-3- (3-diméthylaminopropyl) -carbodiimide (EDC) solide a été ajouté à la suspension à la concentration désirée (1 mM ou 10 mM) ou dans 10 mM EDC à pH 6. Après 90 min d'agitation douce à 25 ° C, une modification à 100% des groupes carboxyliques exposés a été obtenue en utilisant 10 mM EDC et pH 4,75, tandis que 40 - 50% a été modifié en utilisant 1 mM d'EDC ou 10 mM et pH 6. La ficine modifiée a été filtrée et 4 g ont été incubés pendant 24 h dans une solution d'hydroxylamine 0,1 M à pH 7 pour récupérer les tyrosines modifiées (Caraway, *et al.,* 1968). La préparation finale a été filtrée et lavée avec du phosphate de sodium 25 mM à pH 7,5 et avec un excès d'eau distillée.

6.2.2. Modification avec le glutaraldéhyde

Une masse de 8 g de la ficine immobilisée sur glyoxyl -agarose a été mise en suspension dans 80 ml de sodium de phosphate 25 mM à pH 7 contenant 0,03%, 0,1% ou 1% (v / v) de glutaraldéhyde pendant 1 h. Ensuite, le biocatalyseur a été lavé avec de l'eau distillée.

Un g de support a été mis en suspension dans 10 ml de tampon carbonate 25 mM pH 8,5 pendant 24h. L'activité a été suivie en utilisant le BANA pendant la modification . Ensuite, 80 ml d'eau ont été ajoutés et du borohydrure de sodium a été ajouté jusqu'a une concentration de 1 mg / ml, agité pendant 30 minutes et lavé avec un excès d' eau distillée .

6. 2.3 . Amination de l' enzyme libre avec EDC et EDA

La ficine libre a été ajoutée à 50 ml de solution d'EDA 1 M à pH 4,75. De l'EDC solide a été ajouté à la suspension jusqu'à une concentration finale de 10 mM. Il a été rapporté qu'après 90 minutes d'agitation douce à 25 ° C, une modification de 100% de tous les groupes carboxyliques exposés a été obtenue (HOARE , *et al.*, 1967) .

L'enzyme modifiée a ensuite été dialysée à 4 ° C pendant 24 h en utilisant une membrane de dialyse avec une coupure de poids moléculaire de 10 kD . La dialyse a été réalisée dans 2,5 L de tampon phosphate 5 mM, le tampon de dialyse a été changé après 2h, 4h, 10h et 24h. L'immobilisation de la ficine aminée ou non - aminée sur glyoxyl-agarose a été réalisée comme décrit précédemment par SIAR *et al,*. (2017). Dans certains cas, l'enzyme immobilisée à pH 9,0 a été incubée après immobilisation à pH 10,0. Le temps d'immobilisation maximal était de 3 h (valeur optimale trouvée en utilisant l'enzyme non aminée) (SIAR *et al,* 2017). Ensuite, les dérivés ont été filtrés sous vide et lavés soigneusement avec de l'eau distillée. Dans certains cas, la concentration d'enzyme a été augmentée (1, 3, 6 et 10 mg / mL ont été utilisés).

7. Inactivation thermique des préparations de ficine

Les différentes préparations de ficine ont été incubées à 55˚C dans de l'acétate de sodium 50 mM à pH 5, le phosphate de sodium 50 mM à un pH de 7 ou de carbonate de sodium 50 mM à pH 9. Pour l'extrait d'enzyme libre, 1 mg / ml de solution ficine a été préparé en le même tampon et la même température.

Des échantillons ont été prélevés périodiquement et l'activité a été déterminée en utilisant le test BANA comme substrat.

6. Utilisation et réutilisation de la ficine immobilisée dans l'hydrolyse de la caséine

La méthode standard de détermination de l'activité enzymatique utilisant la caséine a été décrite par KUNITZ (1964). Dans certains cas, afin de montrer l'effet de l' Urea sur l'hydrolyse de la caséine nous avon ajoute des concentration en ures a la preparation de caseine. La température a été modifiée (de 55 à 70 ° C). Afin de tester la possibilité de réutilisation de l'enzyme immobilisée dans l'hydrolyse de la caséine, cinq cycles d'hydrolyse de la caséine de 1 h ont été réalisés à 60 et 65 ° C comme décrit ci-dessus. Après 1 h d'hydrolyse, la production de peptide a été vérifiée et l'enzyme immobilisée a été lavée cinq fois avec 10 volumes d'eau distillée, et employée dans un nouveau cycle de réaction.

Pour la ficine-glutaraldéhyde, six cycles d'hydrolyse de la caséine (2 h chacun) ont été effectués à 50 º C et pH 7. Après 2 h de protéolyse de caséine, la production de peptide a été vérifiée comme décrit ci-dessus et l'enzyme immobilisée a été lavée 5 fois avec 10 volumes d'eau distillée et utilisé dans un nouveau cycle de réaction.

9. Utilisation de glyoxyle -agarose immobilisée ficine extrait dans la coagulation du lait

Dans cette partie du travail, la ficine a été immobilisée sur du glyoxyl - agarose. Différentes charges de biocatalyseur ont été utilisées (3, 10, 30 et 85 mg / g) pour montrer l'effet de la charge sur l'activité de coagulation du lait.

9.1. Activité de coagulation de la caséine des biocatalyseurs de la ficine

L'activité coagulante de la ficine immobilisée et libre a été déterminée selon la méthode décrite par PESSELA *et al.* (2004) avec de légères modifications. La caséine de lait bovin a été préparée à 1% (p / v) dans un tampon d'acétate de sodium 50 mM contenant 5 mM de chlorure de sodium à pH 5,6 . Pour démarrer la réaction, la quantité souhaitée de ficine immobilisée ou libre a été ajoutée à 2 mL du substrat (solution de caséine à 1%) à 25 ° C et la réaction a été suivie spectrophotométriquement par l'augmentation d'absorbance à 550 nm favorisée par la précipitation des protéines. L'activité était inversement proportionnelle au point d'inflexion de cette courbe.

9.2. Coagulation de la caséine en deux étapes

L'hydrolyse de la caséine à 1% (p / v) a d'abord été réalisée à 4 ° C pour éviter la précipitation de l'hydrolysat (PESSELA, *et al.* 2004). Des échantillons ont été prélevés périodiquement, chauffés à 40 ° C pendant 20 minutes pour permettre la précipitation de la protéine hydrolysée. Ensuite, l'absorbance a été déterminée à 550 nm.

9.3. Coagulation en deux étapes du lait écrémé

La première étape de la coagulation du lait écrémé (Celta, pH 6), l'enzymatique, a été réalisée à 4 ° C pendant 2,5 h (le volume de lait utilisé était de 40 mL en utilisant 40 mg de ficine sous forme immobilisée soit 300 µg de ficine libre). Ensuite, lors de l'utilisation d'enzymes immobilisées, les échantillons ont été centrifugés pour éliminer le biocatalyseur en profitant de l'absence de précipitation de l'hydrolysat de caséine à basse température. Dans une seconde phase, le surnageant a été chauffé à 40 ° C pendant 2 h (le temps nécessaire pour avoir une séparation claire entre le coagulum et le petit-lait) et la caséine coagulée a été récupérée par filtration (la filtration a été réalisée sur un mouchoir en papier pendant 15 min pour chaque coagulum) et pesé. Le rendement a été calculé selon la formule décrite par JEANTET , *et al* ., (2008) .

***Rendement = (poids de coagulum récupéré / poids de lait utilisé) * 100* .**

9.4. Analyse SDS-PAGE du coagulum

La SDS-PAGE a été réalisée selon LAEMMELI (1970) en utilisant 12% de gels de séparation et 4% d'empilage. Les échantillons ont été mélangés à un rapport de 1 mg de coagulum par m L de l'échantillon tampon. Les autres étapes ont été effectuées comme décrit pour l'extrait de ficine.

RESULTS

The expression immobilized enzymes refer to "enzymes physically confined or localized in a certain defined region of space with retention of their catalytic activities, and which can be used repeatedly and continuously." (BRENA, *et al.*, 2013).

Actually, immobilized enzymes are the topic of considerable interest because of their advantages over soluble enzymes and their use in industrial processes. immobilization persist as a key step in many biocatalysts' developments. Initially developed for facilitating the recovering and reus of the enzymes (Sheldon *et al.*, 2013, liese and HILTERHAUS 2013, DI COSIMO *et al,*. 2013), nowadays, immobilization protocol may be used to improve enzyme stability, activity, resistance to chemicals or inhibitors, in some cases even purity and hyperactivation (MATEO *et al.*, 2007, BARBOSA *et al.*, 2015 and SANTOS *et al.*, 2015).

The most suitable activated supports for enzyme stabilization via multipoint covalent attachment are glyoxyl (MATEO *et al., 2007*), glutaraldehyde (BARBOSA *et al., 2014*), vinylsulfone (DOS SANTOS *et al., 2015*) and epoxyde (MATEO *et al., 2006*).

Thus, in this part of work, we have immobilized extract of ficin in glyoxyl agarose and on amino and amino glutaraldehyde agarose beads following the activity using casein and a small synthetic substrate. Our objective is to obtain a stabilized biocatalyst of immobilized ficin with high activity versus casein that can be used to hydrolyse diverse proteins under different experimental conditions. The results were shows as two published paper. The first one immobilisation of ficin on glyoxyl agarose ´´*Stabilization of ficin extract by immobilization on glyoxyl agarose.Preliminary characterization of the biocatalyst performance in hydrolysis of proteins*´´. The second part is the immobilization of ficine using amino and amino glutaraldehyde agarose ´´*Immobilization/Stabilization of Ficin Extract on Glutaraldehyde-Activated Agarose Beads. Variables That Control the Final Stability and Activity in Protein Hydrolyses*´´

Abstract

A protein extract containing ficin was immobilized on glyoxyl agarose at pH 10 and 25 °C. The free enzyme remained fully active after 24 h at pH 10. However, the enzyme immobilized on the support retained only 30% of the activity after this time using a small substrate. After checking the stability of ficin preparations obtained after different enzyme-support multi-interaction times, it was found that it reached a maximum at 3 h (40-folds more stable than the free enzyme at pH 5). The immobilized enzyme was active in a wide range of pH (e.g., retained double activity at pH 10 than the free enzyme) and temperatures (e.g., at 80 °C retained three-folds more activity than the free enzyme). The activity versus casein almost matched the results using the small substrate (60%) at 55 °C. However, in the presence of 2 M of urea, it became three times more active than the free enzyme. The immobilized enzyme could be reused five cycles at 55 °C without losing activity.

Keywords: Protease stabilization, Protease immobilization, Improved enzyme performance, Ficin, Glyoxyl agarose, Protein hydrolysis.

Resumé

L'immobilisation de la ficine sur le glyoxyl agarose a était réalisé à 25ºC dans le tampon carbonate a pH 10. La ficine libre maintient la totalité de son activité a pH 10 pendant 24h à 25ºC. Cependant, après 24h d'immobilisation la ficine retient seulement 30% de son activité initiale. Apres mesure de stabilité des déférente préparation obtenue après déférents temps de contact enzyme-support. La stabilité maximale est obtenue après 3h de contacte enzyme-support (qui a une stabilité de 40 fois supérieure a celle de l'enzyme libre. L'enzyme immobilisée est très active dans une grande gamme de pH (exemple a pH10 avec une activité double que la ficine libre) et de température (jusqu'à 80 ºC retient 3 fois d'activité que l'enzyme libre) comparé avec l'enzyme libre. L'activité en utilisant ls caséine ou le substrat synthétique est presque la même. En présence de 2M de l'unrea l'activité devient trois fois plus que celle de l'enzyme libre. la ficine immobilisée obtenue peut être réutilisée dans 5 cycle d'hydrolyse des caséine a 55 ºC sans perdre son activité.

Mots clés: *Stabilisation des protéases, Immobilisation des protéases, Amélioration des performances enzymatiques, Ficine, Glyoxyl agarose, Hydrolyse des protéines.*

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Stabilization of ficin extract by immobilization on glyoxyl agarose. Preliminary characterization of the biocatalyst performance in hydrolysis of proteins

El-Hocine Siar[a,b], Hadjer Zaak[a,b,c], Jakub F. Kornecki[a], Mohammed Nasreddine Zidoune[b], Oveimar Barbosa[d], Roberto Fernandez-Lafuente[a,*]

[a] Departamento de Biocatálisis. Instituto de Catálisis-CSIC, Campus UAM-CSIC, Madrid, Spain
[b] Equipe TEPA, Laboratoire LNTA, INATAA, Université des Frères Mentouri Constantine 1, Food Biotechnology Division, Biotechnology Research Center (CRBt), 25000 Constantine, Algeria
[c] Applied Microbiology in Environment Laboratory, Science Department, Ibn Khaldoun University, Tiaret, Algeria
[d] Departamento de Química, Facultad de Ciencias, Universidad del Tolima, Ibagué, Colombia

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ABSTRACT

A protein extract containing ficin was immobilized on glyoxyl agarose at pH 10 and 25 °C. The free enzyme remained fully active after 24 h at pH 10. However the enzyme immobilized on the support retained only 30% of the activity after this time using a small substrate. After checking the stability of ficin preparations obtained after different enzyme-support multi-interaction times, it was found that it reached a maximum at 3 h (40-folds more stable than the free enzyme at pH 5). The immobilized enzyme was active in a wide range of pH (e.g., retained double activity at pH 10 than the free enzyme) and temperatures (e.g., at 80 °C retained three-folds more activity than the free enzyme). The activity versus casein almost matched the results using the small substrate (60%) at 55 °C. However, in the presence of 2 M of urea, it became three times more active than the free enzyme. The immobilized enzyme could be reused five cycles at 55 °C without losing activity.

1. Introduction

The hydrolysis of proteins catalyzed by proteases has a great interest in diverse industrial areas [1–3]. Proteases are used from detergent formulations [4–6] to food biotechnology process (e.g., production of aminoacids or active peptides using residual products) [7–15] or to improve digestibility, texture or taste of foods [16]. The enhancement of antioxidant capability [17] or reduction in allergenic compounds are other possible effects of protease treatment of foods [18,19]. The advantages compared to chemical processes are clear, as specific proteases may perform some modifications of food that are not possible using standard chemical processes, that will be neither selective nor specific and may drive to aminoacid chemical modification [1]. This variety of applications requires proteases to be stable and active under a wide range of conditions.

Proteases are relatively expensive and the reuse of the enzymes seems necessary to get economically suitable processes [1]. Protease immobilization is the most suitable tool to facilitate its reuse provided that the immobilized enzyme is stable enough [20,21]. Considering this fact, it seems adequate to couple the improvement of other enzyme features to its immobilization. Thus, an intense research in this area has been developed and has shown that a proper immobilization may have some further positive effects on many enzyme features. For example if multipoint covalent attachment is achieved, the enzyme structure becomes more rigid and enzyme stability under any distorting reagent may increase [22–25]. Immobilized enzymes have in some instances an improved activity, selectivity or specificity due to conformational changes caused by immobilization [26] or thanks to enzyme stabilization that permit to keep the enzyme conformation under more drastic conditions, preventing aggregation, unfolding, etc. [27]. In some instances, enzyme immobilization may be coupled to enzyme purification [28].

In the case of proteases, immobilization on porous supports will have both additional problems and advantages compared to other enzymes. A clear advantage is that an immobilized protease molecule cannot be attacked by other immobilized protease molecule (this protection does not occur using non-porous materials). Thus inactivation by enzyme proteolysis may not occur [29,30].

The main problems are namely two, both related to the size of the substrate. If the substrate is insoluble (e.g., a textile, or a protein

* Corresponding author at: Roberto Fernandez-Lafuente, ICP-CSIC; C/Marie Curie 2, Campus UAM-CSIC, Cantoblanco 28049, Madrid, Spain.
E-mail address: rfl@icp.csic.es (R. Fernandez-Lafuente).

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aggregate), an enzyme immobilized on a porous support will not be able to access it [29]. This problem does not exist using non-porous materials [30]. The protein aggregates may be solubilized using urea and that way become a substrate for enzyme immobilized inside a pore. However, the hydrolysis of these urea-solubilized proteins requires having an immobilized protease able to maintain its function under these drastic conditions. Additionally, enzyme activity may be destroyed as in any other protein due to conformational changes caused by the immobilization. However, using proteases, even proteases with the active center fully preserved may be functionally inactive, because only properly oriented enzyme molecules will be able to hydrolyze large proteins [31]. Enzyme molecules with the active center oriented toward the support surface will be inactive versus large proteins. In fact, when the enzyme orientation is not completely looking toward the outside, enzyme activity may be reduced by steric hindrances if immobilized proteases molecules are close one to each other [29]. For this reason, checking the enzyme activity versus a small and a large substrate may be convenient when working with these enzymes.

Vegetable proteases have been used since ancient times. For example, the latex of fig tree has been used for the manufacture of cheese [32–37], in the brewing [38] and pharmaceutical [33,39] industries, for meat tenderization [40,41] and in the production of bioactive peptides [42]. One specific application of ficin is the production of fragment of antibodies via specific hydrolysis of some peptide bonds, as it is considered that ficin gives a more rapid and reproducible hydrolysis map than using other proteases [43,44].

These fig tree latex uses are founded in the presence in the fluid of a protease activity, called ficin (EC 3.4.22.3). Ficin isoforms are glycoproteins having a high similarity of sequence with bromelaine [45]. Its active center consists of two aminoacids: the catalytic cysteine and a histidine [33,46,47]. Autolysis of ficin gives several peptides with molecular weights ranging from 14 to 18 KDa [39]. Four isoforms of this enzyme have been described (A, B, C and D) [48] and crystallized, resolving their structure [47] (see supporting information Figures 1S–4S). Ficin has a high thermal stability (optimal temperature range from 67 to 82 °C [32–34,41,49,50]), although Grozonka et al. [40] reported the optimal temperature lied in the range between 45 and 55°C. These changes on optimal T depend on the exact reaction conditions and substrate utilized. Ficin extract has been successfully employed in a range of pH from 5 to 8.5 [32,33,40,45].

Glyoxyl activated supports are very adequate to permit an intense multipoint covalent attachment between the enzyme and the support and that way, to obtain a more rigid protein molecule, and very likely an improved stability [51]. Due to the reversibility of the individual imino bond, in the absence of imino bond stabilizers (e.g., thiolated compounds [52]), enzyme immobilization is only produced when several enzyme-support bonds are formed [53,54]. This way, immobilization is directed to the area of the protein surface where it is most likely to have an intense multipoint covalent attachment. However, this immobilization method has two problems [51,53]. The first one, immobilization needs to be performed at alkaline pH value (to have Lys groups in unprotonated form). The second one, it is necessary a final reduction with sodium borohydride, and that may affect some proteins [55].

As support we have utilized agarose due to its very good properties to get a stable enzyme, mainly inertness, activation possibilities and easy handling in the laboratory [56]. The support is resistant to mechanical stirring although it may be easily milled under magnetic stirring [57]. As model protein substrate, we have selected casein that may have also a real interest [10,58].

Thus, in this article, we have immobilized extract containing ficin in glyoxyl agarose beads following the activity using casein and a small synthetic substrate. There are scarce and quite old examples of immobilization of this enzyme [32,59–62], although the company Thermus commercialized an agarose-immobilized ficin for hydrolysis of antibodies [43,44]. The Cys residue in the active center and the glycosylate nature of the enzyme may promote some problems for the enzyme stabilization via multipoint covalent attachment. Our objective is to obtain a stabilized biocatalyst of immobilized ficin with high activity versus large proteins that can be used to hydrolyze diverse proteins under different experimental conditions.

2. Materials and methods

2.1. Materials

Benzoyl-arginine-p-nitroanilide (BANA), cysteine and casein were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Agarose beads 4BCL support was purchased from Agarose Bead Technologies (ABT), 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 was prepared as previously described [53,54].

2.2. Preparation of *fi*cin extract samples

Fresh fig latex was collected by breaking the immature green fruits and leaves of F. carica L. growing in Kabylia, a Mediterranean region (Adekar, Bejaia), and collecting the fluid in a clean flask at 4 °C. All the latex samples used in this study were collected freshly from the fig tree in June 2016 and used 4 months later as maximum. The latex was centrifuged at 3200 g for 15 min at 4 °C [34] to remove gums and other debris. The insoluble material was discarded and the supernatant was retained and represented the "crude extract of ficin" (with a concentration of 89 mg protein/mL), stored at −20 °C until use. The protein concentration was quantified by Bradford's method [63]. BSA was used as the standard protein. The extract was not further characterized, but presence of inhibitor can be discarded as the activity was proportional to the amount of soluble enzyme (this will increase the concentration of any likely inhibitor). Using immobilized enzyme, the molecules will be dispersed on the support and washed, thus, the incidence of inhibitors of other molecules on enzyme properties may be discarded [27].

2.3. Enzymatic assays

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

For standard assays, 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 at 25 °C [57]. The hydrolysis rate was determined by continuously measuring the p-nitroaniline formed at 405 nm (ε 8800 for p-nitroaniline under these conditions). Activity of ficin was expressed as the amount of p-nitroaniline formed by 1 mg of protein per min. In some instances, the pH was altered or the temperature increased (the corresponding ε were calculated under these new conditions).

The standard method of enzyme activity determination using casein was described by Kunitz [64] and here it has been used with some slight modifications. 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. In some instances, the temperature was altered (from 55 to 70 °C), or urea to a concentration of 2 M was added. To 1 mL of substrate solution, 100 μg 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 protein but the peptides are maintained 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

1 h incubation in TCA. One unit of activity is defined as the amount of enzyme that increases the absorbance by 0.001 min^{-1} under the given assay conditions.

2.4. Immobilization of *fi*cin extract on glyoxyl-support

Ficin extract immobilization was performed by adding 10 g of glyoxyl agarose to 100 mL of ficin extract solution (protein concentration was 1 mg/mL) prepared in 50 mM sodium carbonate at pH 10.0 at room temperature under continuous stirring. A loading of 10 mg protein per g of support loading (maximum loading of this support is 30 mg/g) was selected to ensure a proper study of the effect of the immobilization in the enzyme properties without diffusion problems [27]. As a reaction end point, derivatives were reduced by addition of solid $NaBH_4$ to reach a concentration of 1 mg/mL [53]. After gentle stirring for 30 min at room temperature, the resulting derivatives were washed with abundant distilled water to eliminate residual sodium borohydride. Immobilization yield is defined as the percentage of offered enzyme that become immobilized on the support, while expressed activity is defined as the ration between observed activities and theoretical immobilized activities.

2.5. Thermal inactivation of *fi*cin preparations

The different ficin preparations were incubated at 55°C in 50 mM sodium acetate at pH 5, 50 mM sodium phosphate at pH 7 or 50 mM sodium carbonate at pH 9. Samples were periodically withdrawn and the activity determined using the BANA assay described above. Stabilizations were calculated from the ratio between the half-lives obtained from the inactivation courses.

2.6. Reuse of the immobilized *fi*cin in the hydrolysis of casein

In order to test the possibility of the reuse of the immobilized enzyme in the hydrolysis of casein, five cycles of casein hydrolysis of 1 h were performed at 60 and 65 °C as described above. After 1 h of hydrolysis, the peptide production was checked and the immobilized enzyme was washed five times with 10 volumes of distilled water, and employed in a new reaction cycle.

3. Results

3.1. Immobilization of *fi*cin extract on glyoxyl agarose beads

Fig. 1 shows the immobilization course of ficin on glyoxyl agarose at pH 10, where it is possible to visualize that immobilization yield is 100%. Under these conditions, the activity of the free ficin extract very slowly decreased over time (around 5% after 24 h), suggesting that enzyme inactivation is not a relevant problem under these conditions (Fig. 1). Full immobilization of ficin extract activity (> 95%) was obtained after 1 h. Although this seems a rapid immobilization, under similar conditions, other enzymes like penicillin G acylase [65], trypsin [66] or chymotrypsin [67] were immobilized in this support in less than 30 min. As immobilization in glyoxyl supports is very depended on the superficial density of reactive groups in the protein [68] and in the support [53], this result suggested that there is not an area of the enzyme surface with a high density of Lys groups (in fact, Table 1 shows the number of Lys of the four isoforms of ficin and Figs. 1S–4S the position of the Lys groups on the proteins surface).

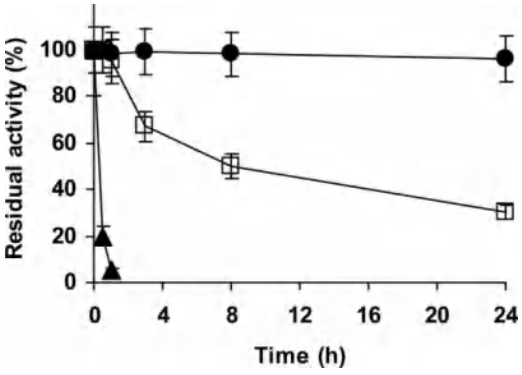

Fig. 1. Immobilization course of ficin in glyoxyl-agarose. Immobilization was performed at pH 10 and 25 °C using 10 g of support and 100 mL of 1 mg/mL ficin (determined by Bradford using BSA as reference). Other specifications are described in methods. Circles: free enzyme under identical conditions; Squares: suspension. Triangles: supernatant.

Table 1
Amino acid composition of the different ficin isoforms.

Isoform	Ficin A	Ficin B	Ficin C	Ficin D2
Amino acid	Number of residues			
Asp	10	9	9	17
Glu	14	12	12	14
Ser	34	17	17	20
Gly	46	22	22	20
His	4	2	2	2
Arg	16	8	8	7
Thr	16	14	13	12
Ala	42	17	17	21
Pro	12	8	8	9
Tyr	24	11	11	11
Val	38	22	22	26
Met	2	2	2	3
Cys	18	7	8	8
Ile	20	13	13	9
Leu	32	11	11	9
Phe	8	3	3	2
Trp	12	6	6	6
Lys	22	14	15	16
Total	370	198	199	212

However, expressed enzyme activity decreased after the first immobilization. After 3 h, it was around 60% of the initial value and after 24 h, the expressed activity was 30%. The reduction step had not effect on expressed enzyme activity. This means that the enzyme was distorted by the reaction with the support.

3.2. Stability of the immobilized preparation

Fig. 2 shows the inactivation courses under different pH conditions of the immobilized enzyme that has been incubated for different times before reduction (1, 3, 8 or 24 h) compared with that of the free enzyme. All immobilized enzymes were far more stable than the free enzyme (around 40-folds at pH 5). It may be expected that a longer incubation time may favor the formation of more enzyme/support bonds (as indicated by the decrease in enzyme activity when the free enzyme is almost fully stable) and that should produce higher enzyme stabilization. However, our results pointed out that the maximum stabilization was obtained after 3 h of immobilization, at pH 7 differences between 1 and 3 h are very short, while at pH 5 are clearer. Free enzyme is more stable at pH 7, then 9 and finally 5, while immobilized preparations differences in stability with the pH are not so relevant (thus, stabilization is shorter at pH 7, just a five-folds). Longer enzyme/support incubation before reduction produced a slight decrease in the final stability of the ficin immobilized preparations, and that is clearer in inactivations at pH 7 and 9 than at pH 5 (Fig. 2). This slightly negative effect on enzyme stability of the incubation time could be related to some conformational changes that could affect the enzyme stability (e.g., by exposing some key group to the medium). Figs. 1S–4S show the distribution of the Lys groups on the enzyme surface, suggesting that there is not an area very rich in Lys for any of the isoforms, except for ficin D2 (where 6–7 Lys groups were near each

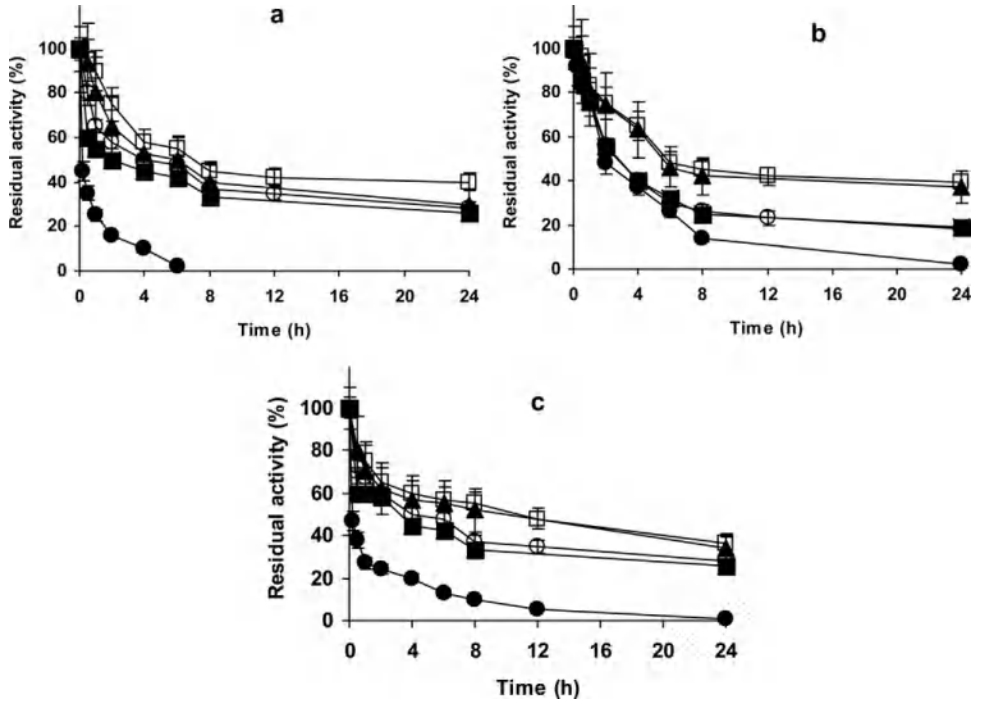

Fig. 2. Effect of immobilization time in the stability of the different ficin preparations at 55 ºC and pH 5 (a), pH 7 (b) or pH 9 (c). Experiments were performed as described in methods using BANA as substrate. Solid Circles: free enzyme; Solid Triangles: 1 h of immobilization, Empty squares: 3 h of immobilization; Empty circles: 8 h of immobilization; Solid Squares: 24 h of immobilization.

other). Thus, an immobilization time of 3 h was selected as the optimal one, permitting to get over 60% of the initial activity and stabilization factors of around 40-folds compared to the free enzyme at pH 5, a very stable one. As a future possibility, the chemical amination of the enzyme to favor a more intense multipoint covalent attachment under milder conditions may be a good way to improve these results [69–72], as all enzyme isoforms have more Glu and Asp than Lys residues (see Table 1). The stabilization values obtained using glyoxyl supports with other proteases are clearly higher (e.g., thousands folds using trypsin or chymotrypsin) [55,73,74]. In general, longer incubation time use to increase enzyme stability because permits the formation of more enzyme support-enzyme bonds [74]. The effect of incubation time found in this paper suggest that some distortions may play a negative role not only on enzyme activity, but in its stability, reducing the impact of the multipoint covalent attachment on enzyme stability (perhaps, due to the oxidable thiol catalytic group). In any case, this is not a complete exception, as some similar results have been reported in other cases, for example immobilizing carboxy peptidase A on glyoxyl agarose, although in this case the decrease in stability started after 48 h of incubation [75]. Even although not very high, the stabilization factor obtained in this research was much higher than the three-folds improved stability reported in other papers for immobilized ficin [59].

3.3. Activity versus BANA of free and immobilized *fi*cin under di*ff*erent conditions

The effect of the pH on the activity of the free and optimal immobilized ficin extract versus BANA has been studied. Maximum activity was found for both preparations at pH 7 (Fig. 3). The decrease in activity was milder for the immobilized enzyme at more alkaline pH value (e.g., at pH 10 the immobilized enzyme maintained around 60% of the activity at pH 7, while the free enzyme activity was under 30% of the maximum at pH 7) and similar at more acid pH value. That means that at alkaline pH value, more drastic conditions for the enzyme, the specific activity of the immobilized enzyme was higher than that of the free enzyme, very likely thanks to the stabilization achieved.

The maximum activity at pH 7 is obtained at 70 °C for both the free enzyme and the immobilized preparation (Fig. 3). However, the higher stability of the immobilized enzyme permitted to retain almost 90% of that activity at 80 °C, while the free enzyme retained only less than 30%. This higher activity of the immobilized enzyme at temperature over the optimal agrees with the data on enzyme stability obtained above.

3.4. Activity of free and immobilized *fi*cin versus casein

The immobilized enzyme presented an activity that was around 60% of that of the free enzyme when assaying the enzyme activity versus the large substrate casein at 55 °C, very similar to the values obtained using the small BANA. At 55 °C, the amount of peptides obtained using free or immobilized enzyme was very similar (results not shown). This suggested that the ficin molecules were properly oriented, with the active center oriented toward the medium and permitting the access to the casein molecules to the active center of the immobilized enzyme. A complete kinetic study may have some complications, as a change in casein concentration may induce some protein-protein interaction that could alter the substrate availability for the immobilized enzymes, but it should be carried out with the optimal catalyst and substrate before any industrial implementation.

However, when the reaction conditions were more drastic, the activity and more interestingly, the amount of peptides obtained using the immobilized enzyme surpass the results obtained with the free enzyme in some instances.

Thus, using both ficin preparations at 60 °C (Fig. 4a) the initial activity was still higher for the free enzyme than for the optimal biocatalyst. However while the reaction using the free enzyme seemed to reach a maximum after only 60 min, the immobilized enzyme permitted to get almost 20% more peptides after 180 min of reaction. This was even clearer at 65 °C, where the reaction catalyzed by free enzyme was fully stopped after 40 min while the immobilized biocatalyst continues hydrolyzing casein for 180 min giving 25% more peptides (Fig. 4b), even though the initial activity still was higher for the free enzyme. In the presence of 2 M urea at 55 °C, the immobilized

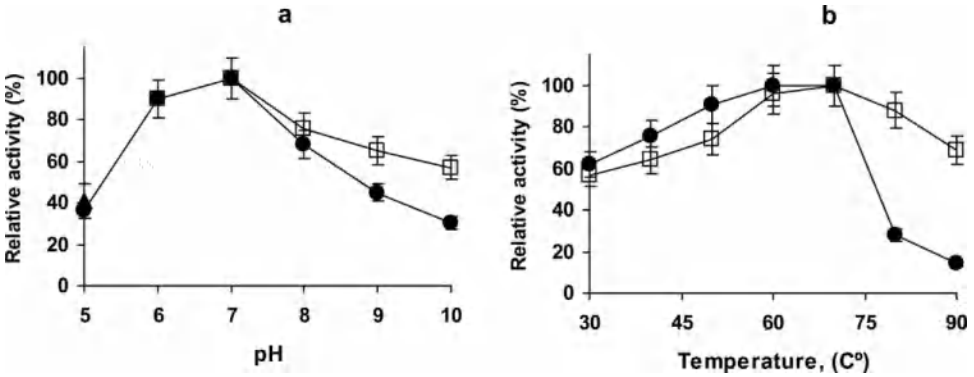

Fig. 3. Effect of the pH (a) and the Temperature (b) on the activity of free (circles) and optimal immobilized (squares) ficin versus BANA. The pH effect on activity was studied at 25 ºC, the effect of T at pH 7. Experiments were performed as described in methods.

enzyme was three-folds more active than the free enzyme, in fact the immobilized enzyme increased the initial activity (by a 30–40%), perhaps as consequence of a higher susceptibility of partially unfolded casein molecules for hydrolysis. However, the free enzyme suffered a drastic decrease in its activity under these conditions (Fig. 4c). The immobilized enzyme permitted to reach almost a 25% more concentration of peptides than the free enzyme after 4 h of reaction (Fig. 4c).

These results exemplified the advantages of the use of the new immobilized biocatalyst, thanks to the improved stability the concentration of peptides formed was higher, and even the activity may become higher than using the free enzyme (without implying a better conformation of the active center, but a higher resistance to unfavorable conditions) [27].

3.5. Reuse of immobilized *ficin*

The main objective of immobilizing an enzyme is to facilitate its reuse. Thus, we evaluated the immobilized enzyme reuse under diverse conditions. Fig. 5 shows that the immobilized ficin may be reused five times in hydrolysis of casein at 55 ºC and pH 7 with a marginal decrease in activity (under 5%). When the enzyme was utilized at 60 ºC, a progressive decrease in the enzyme activity could be noted after each reaction cycle, the immobilized enzyme only retaining around 65–70% of the initial activity after five reuses. That way, a temperature of 55 ºC

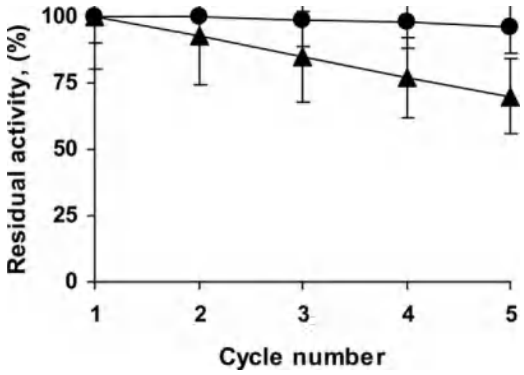

Fig. 5. Operational stability of optimal immobilized ficin preparation in hydrolysis of casein at pH 7 and 55 ºC (circles) or 60 ºC (triangles). Experiments were performed as described in methods.

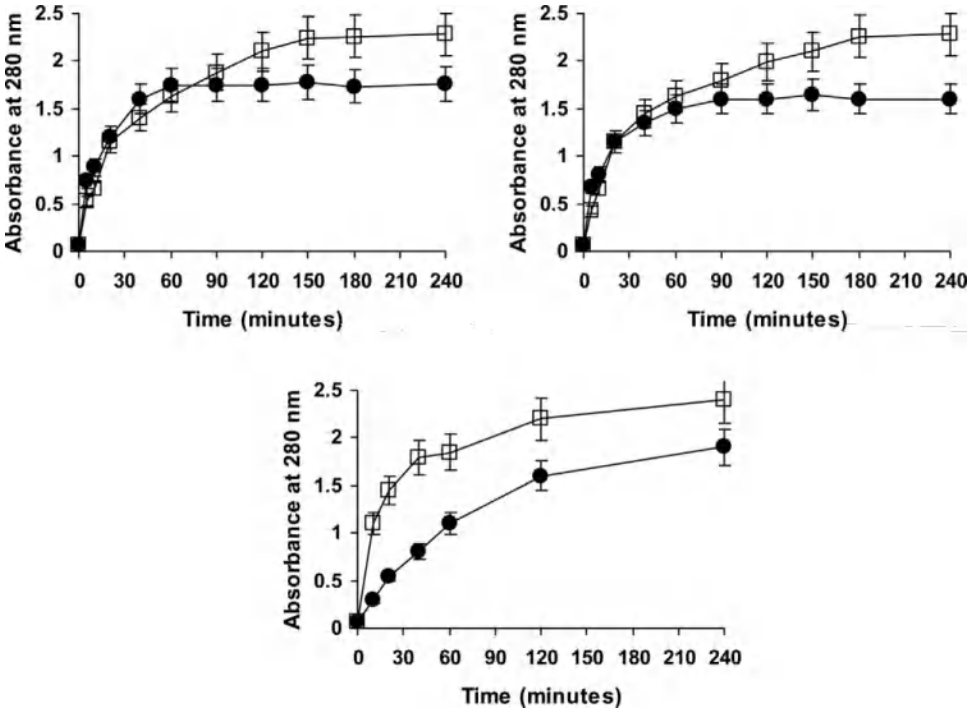

Fig. 4. Increase in absorbance at 280 nm obtained in the hydrolysis of casein at pH 7 and 60 ºC (a), 65 ºC (b) or in the presence of 2 M urea at 55 ºC (c) catalyzed by free (circles) and immobilized (squares) ficin. Experiments were carried out as described in methods.

seemed to be recommended for the use of this ficin preparation in hydrolysis of proteins.

4. Conclusion

This article shows the preparation of an immobilized biocatalyst of ficin with a significant stabilization (40-folds) compared to the free enzyme. The enzyme seems to be immobilized in a proper orientation to recognize large substrates, exhibiting more than 60% of the initial activity versus both the small BANA and the large casein. Enzyme/support reaction times that were longer than 3 h produced a decrease in enzyme stability, perhaps related to a conformational change that favors some modification of a critical group in enzyme activity (e.g., the catalytic Cys). This stabilization permitted that the enzyme may be used under more drastic conditions in hydrolysis of casein (e.g., higher temperature or presence of urea).The operational stability of the immobilized enzyme at 55 ºC and pH 7 seems very adequate for the application of this biocatalyst in hydrolysis of proteins. Further studied should be carried out using analyzing the enzyme of the enzyme load in enzyme activity for each likely application of the immobilized ficin. High enzyme loading may produce further steric hindrance for large substrates, making that optimal loading may be different for each application [27]. The fact that immobilization is not very rapid, may permit that the enzyme stability may be similar using high or low loading, as a close packing may be not expected [76,77].

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.procbio.2017.04.009.

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Conclusion

This article shows the preparation of an immobilized biocatalyst of ficin with a significant stabilization (40-folds) compared to the free enzyme. The enzyme seems to be immobilized in a proper orientation to recognize large substrates, exhibiting more than 60% of the initial activity versus both the small BANA and the large casein. Enzyme/support reaction times that were longer than 3 h produced a decrease in enzyme stability, perhaps related to a conformational change that favours some modification of a critical group in enzyme activity (e.g., the catalytic Cys). This stabilization permitted that the enzyme may be used under more drastic conditions in hydrolysis of casein (e.g., higher temperature or presence of urea). The operational stability of the immobilized enzyme at 55 °C and pH 7 seems very adequate for the application of this biocatalyst in hydrolysis of proteins. Further studied should be carried out using analyzing the enzyme of the enzyme load in enzyme activity for each likely application of the immobilized ficin.

High enzyme loading may produce further steric hindrance for large substrates, making that optimal loading may be different for each application [27]. The fact that immobilization is not very rapid, may permit that the enzyme stability may be similar using high or low loading, as a close packing may be not expected [76,77].

Conclusion

Cet article montre la préparation d'un biocatalyseur de ficine immobilisé avec une significative stabilisation (40 fois) par rapport à l'enzyme libre. L'enzyme semble être immobilisée dans une orientation appropriée pour reconnaître les grands substrats, gardant plus de 60% de l'activité initiale par rapport BANA et à aux caséines. Les temps de contacte enzymatique/support plus que 3 h ont produit une diminution de la stabilité de l'enzyme, peut-être liée à un changement de conformation qui favorise certaines modifications de groupe essentiel pour l'activité enzymatique (par exemple, le Cys catalytique). Cette stabilisation a permis utilisation de l'enzyme dans des conditions plus drastiques dans l'hydrolyse de la caséine (par exemple, une température plus élevée ou la présence d'urée). La stabilité opérationnelle de l'enzyme immobilisée à 55 ° C et pH 7 semble très adéquate pour l'application de ce biocatalyseur dans l'hydrolyse des protéines. Une étude plus approfondie devrait être effectuée pour analyser l'effet de la charge sur l'activité enzymatique pour chaque application probable de la ficine immobilisée.

Une charge enzymatique élevée peut produire un encombrement stérique supplémentaire pour les grands substrats, ce qui fait que la charge optimale peut être différente pour chaque application [27]. Le fait que l'immobilisation ne soit pas très rapide peut permettre une stabilité similaire en utilisant une charge élevée ou faible, car un compactage serré peut ne pas être obtenue [76,77].

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

Résumé

L'extrait de ficine a été immobilisé sur différentes agarose aminé. L'immobilisation par des changements ioniques a donné un très faible rendement avec une activité résiduelle de 10% de l'activité initiale et aucun effet sur la stabilité de l'enzyme. Le traitement avec le glutaraldéhyde de cette enzyme immobilise provoque une inactivation totale. L'utilisation de support amine et active avec le glutaraldéhyde a donné une immobilisation optimale à pH 7 (à pH 5 le rendement d'immobilisation était de 80% et a pH 9 l'enzyme immobilise est inactivée). A pH 7 une immobilisation totale est obtenue avec 40 % d'activité résiduelle avec le BANA et 30 % avec les caséines. Une stabilité de la ficine peut être observe après immobilisation mais cette stabilité dépend de pH d'inactivation et de substrat utilisée. La charge maximale de support en enzyme était de 70 mg enzyme/g support et cette charge n'affecte pas l'activité en utilisant le substrat synthétique mais affect significativement l'activité en utilisant les caséines. A partir de 30mg / g l'activité diminue cette diminution est due probablement à la présence de particule enzymatique proche l'une de l'autre empêchant la pénétration des caséines.

***Mots clés**: immobilisation au glutaraldéhyde; polyvalence du glutaraldéhyde; problèmes stériques d'activité enzymatique; effet de la charge sur l'activité enzymatique*

catalysts

MDPI

Article

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

El-Hocine Siar [1,2,†], Sara Arana-Peña [1,†], Oveimar Barbosa [3], Mohammed Nasreddine Zidoune [2] and Roberto Fernandez-Lafuente [1,*]

1 Departamento de Biocatálisis, Instituto de Catálisis-CSIC, Campus UAM-CSIC, 28049 Madrid, Spain; hocines1@hotmail.fr (E.-H.S.); sara_arana@hotmail.com (S.A.-P.)
2 Equipe TEPA, Laboratoire LNTA, INATAA, Université des Frères Mentouri Constantine 1, 25000 Constantine, Algeria; zidounem@yahoo.fr
3 Departamento de Química, Facultad de Ciencias, Universidad del Tolima, 730006299 Ibagué, Colombia; oveimar@gmail.com
* Correspondence: rfl@icp.csic.es; Tel.: +34-915-854-941
† Both authors have evenly contributed to this paper.

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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.

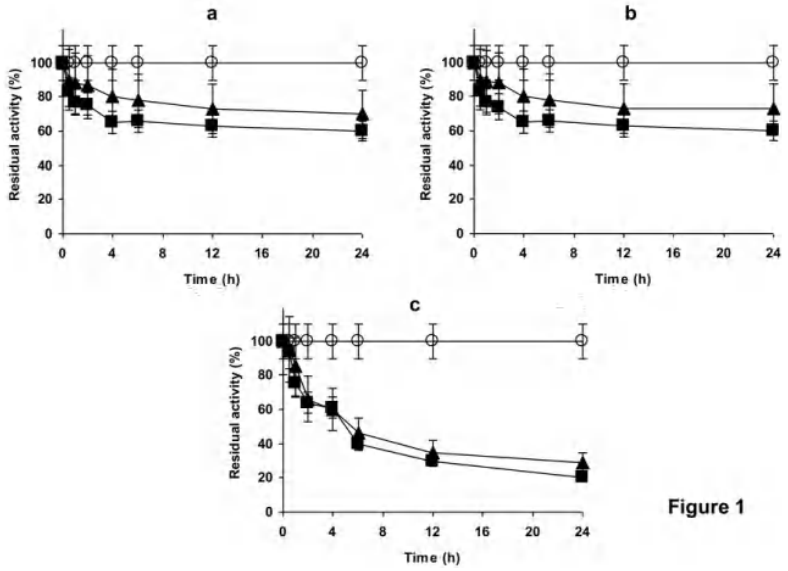

Figure 1. Immobilization of ficin extract on MANAE-agarose beads at pH 5 (**a**), 7 (**b**) or 9 (**c**). Immobilization was performed as described in methods. Activity was followed using BANA as substrate. Circles: reference; Triangles: suspension, Squares: supernatant.

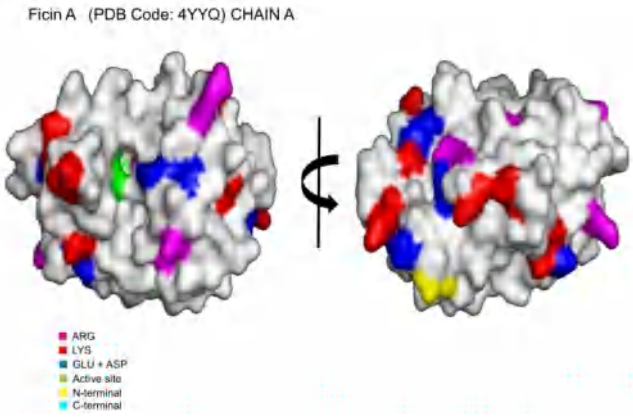

Figure 2. 3D surface structure model of Ficin A obtained from the Protein Data Bank (PDB) and displayed using PyMol versus 0.99. Arg, Lys, Glu+Asp, -NH_2 terminal and -COOH terminal residues are indicated.

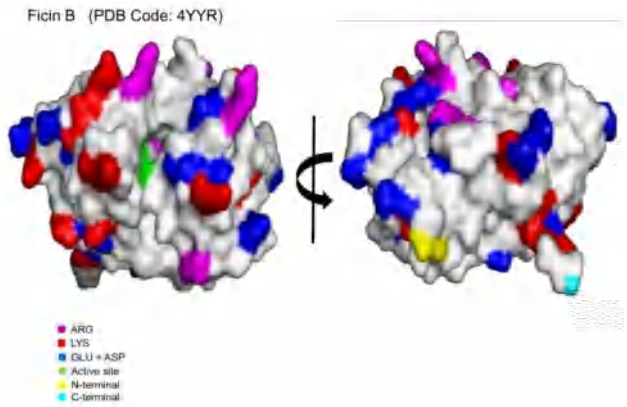

Figure 3. 3D surface structure model of Ficin B obtained from the PDB and displayed using PyMol versus 0.99. Arg, Lys, Glu+Asp, -NH_2 terminal and -COOH terminal residues are indicated.

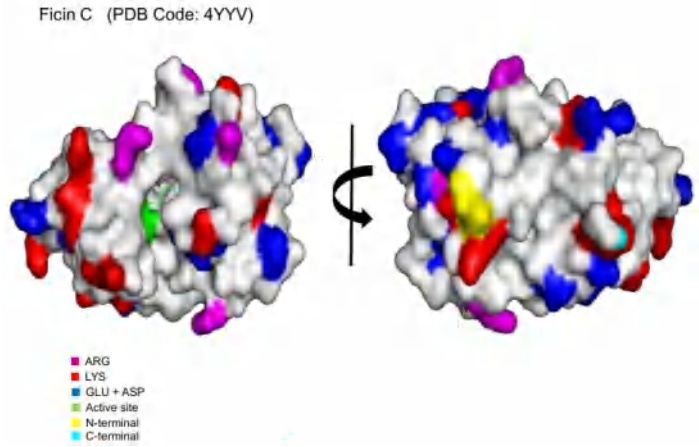

Figure 4. 3D surface structure model of Ficin C obtained from the PDB and displayed using PyMol versus 0.99. Arg, Lys, Glu+Asp, -NH_2 terminal and -COOH terminal residues are indicated.

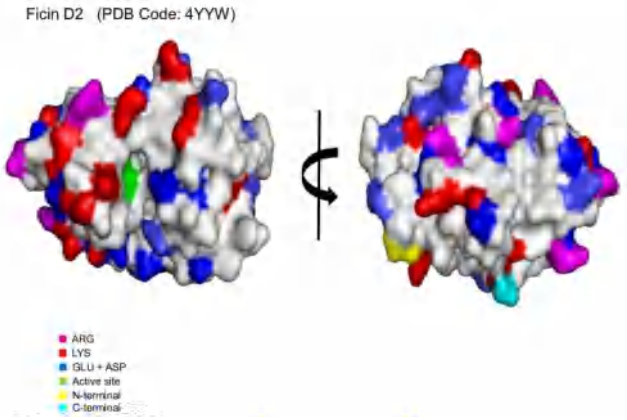

Figure 5. 3D surface structure model of Ficin D_2 obtained from the PDB and displayed using PyMol versus 0.99. Arg, Lys, Glu+Asp, -NH_2 terminal and -COOH terminal residues are indicated.

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.

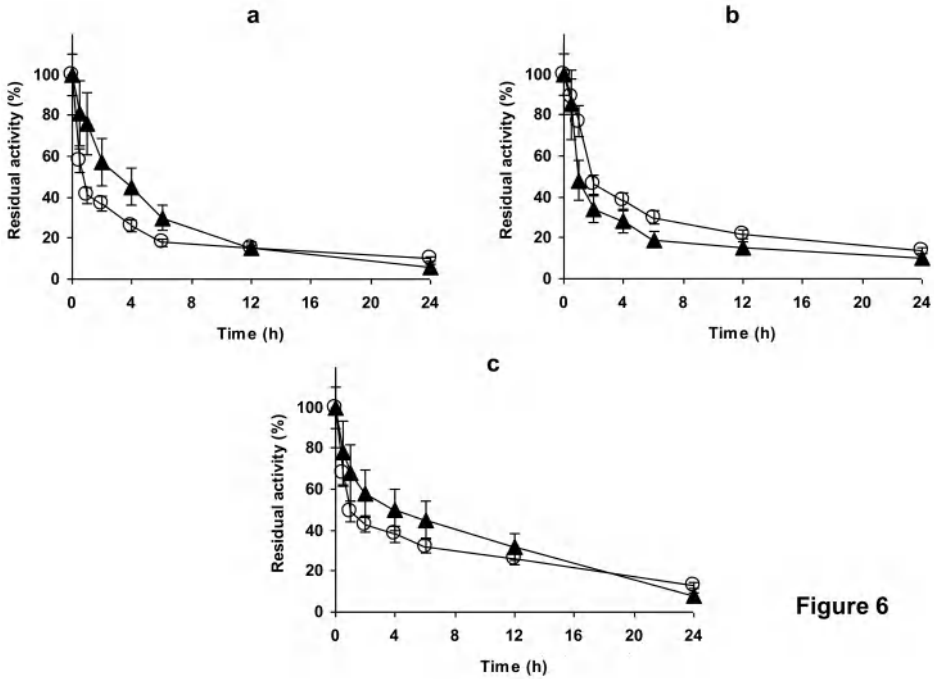

Figure 6. Inactivation courses of the ionically exchanged ficin extract on MANAE-agarose beads at pH 7. The inactivation studies were performed as indicated in Methods section at pH 5, (**a**), 7 (**b**) or 9 (**c**) at 55 °C, using BANA as substrate. Circles: Free enzyme. Triangles: immobilized enzyme.

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.

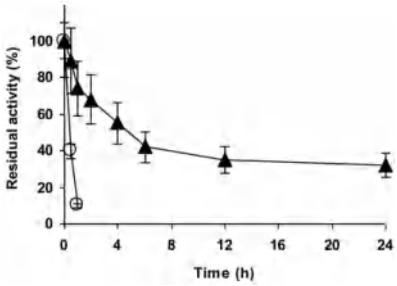

Figure 7. Modification of glyoxyl (triangles) or MANAE agarose (circles) immobilized ficin extract when incubated in 1% glutaraldehyde at pH 7. Experiments were performed as described in Methods using BANA as substrate.

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 that versus BANA.

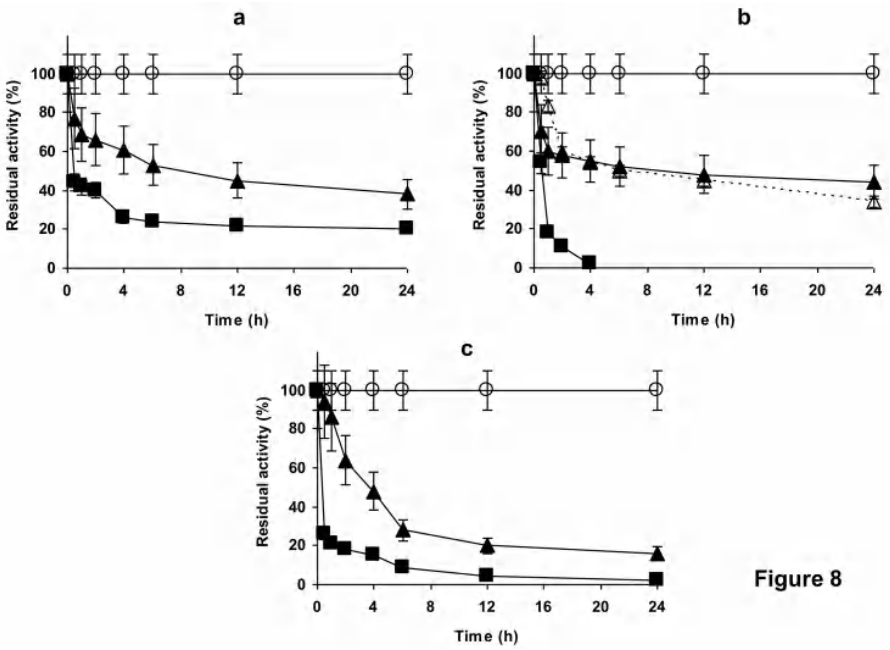

Figure 8. Immobilization of ficin extract on MANAE-agarose beads activated with glutaraldehyde. Experiments were performed as described in the Section 3 using 25 mM of buffer, at pH 5 (**a**), 7 (**b**) or 9 (**c**). Open circles, solid lines: reference; Using BANA as substrate (solid lines, solid symbols):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

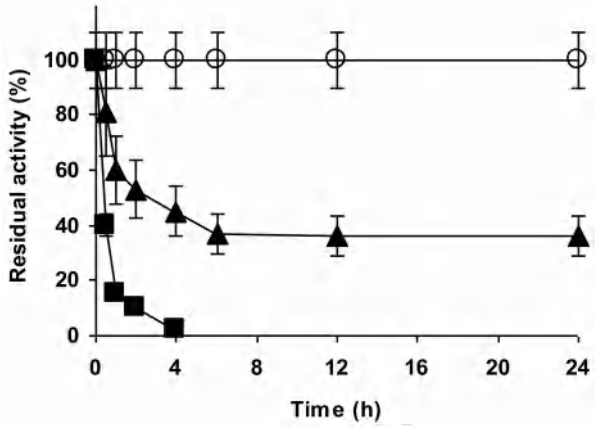

Figure 9. Immobilization of ficin extract on MANAE-agarose beads activated with glutaraldehyde at pH 7 and 200 mM sodium phosphate using BANA as substrate. Experiments were performed as described in Methods, using BANA as substrate. Circles: reference; Triangles: suspension, Squares: supernatant.

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.

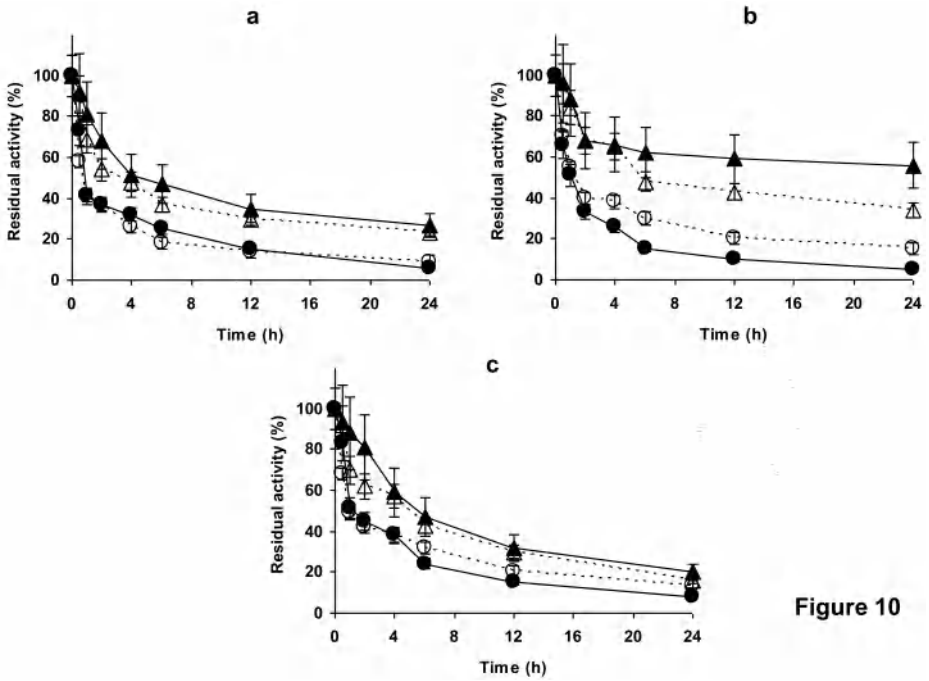

Figure 10. Inactivation of ficin immobilized at pH 7 using 25 mM sodium phosphate. Experiments were performed as described in Section 3, at pH 5 (**a**), 7 (**b**) or 9 (**c**) and 55 °C. Solid lines and solid circles: inactivation was followed using BANA, Dotted line and empty symbols: inactivation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

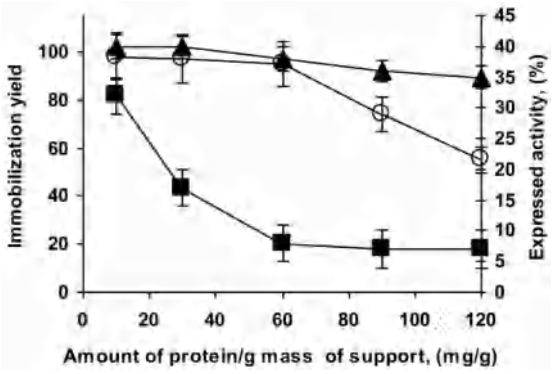

Figure 11. Immobilization yield and expressed activity when growing the amount of offered ficin extract per g of MANAE activate with glutaraldehyde support. Experiments were carried out as described in Methods section. Triangles: immobilization yield; Circles: expressed activity versus BANA, Squares: 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× *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 °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 ± 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 ε for *p*-nitroaniline was 8800). Activity of ficin was expressed as μmols 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 μL 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.

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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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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.

Conclusions

L'extrait de ficine peut être immobilisé sur des supports activés par le glutaraldéhyde, les meilleurs résultats en termes de rendement d'immobilisation et d'activité exprimée sont obtenus lorsque l'enzyme est immobilisée à pH 7.

L'immobilisation dans le tampon phosphate de sodium à 25 ou 200 mM est relativement similaire, tandis que le support aminé est incapable d'immobiliser plus de 30 à 40% de l'enzyme. Cela suggère que la première étape d'immobilisation est dans les deux cas l'attachement covalent de l'enzyme. L'immobilisation à pH 5 n'a pas permis une immobilisation complète de l'enzyme, tandis qu'à pH 9, l'enzyme est inactivée. La stabilisation dépendait du pH et du substrat utilisé pour déterminer l'activité résiduelle, étant plus grande avec le BANA et moins avec la caséine. Les résultats suggèrent un réseau complexe d'interactions entre l'enzyme et le support qui affectent différemment l'activité par rapport aux différents substrats. Pour l'hydrolyse de la caséine, l'activité spécifique de l'enzyme diminue rapidement avec des charges élevées, alors qu'elle est presque identique avec BANA. La charge enzymatique n'a pas d'effet significatif sur la stabilité de l'enzyme immobilisée.

Nowadays there are many different methods to improve enzyme properties, for example multipoint covalent immobilization (MATEO *et al,*. 2007, SHELDON and PELT 2013, GARCIA-GALAN *et al,*. 2011), or chemical modifications of the enzymes (SPICER, and DAVIS 2014, BOUTUREIRA, and BERNARDES 2015), microbiology (D'AMICO *et al.,* 2003), molecular biology (EIJSINK *et al,*. 2005, CHALKER *et al,*. 2009).

There are two different possibilities to modify the enzyme, using the immobilized or the free enzyme. For the immobilized enzyme chemical modification have for objectives to improve stability of the biocatalyst. This stabilization can be obtained following different strategies such as amination using ethylenediamine (EDA) or using glutaraldehyde the create an internal cross linking (enzyme-support or enzyme-enzyme). For the free enzyme the more used modification is the amination using EDA.

In this work we have carried out both chemical modification on the immobilised enzyme and on the free enzyme and we have published the results in two scientific articles. The first one ´´ *Solid phase chemical modification of agarose glyoxyl-ficin: Improving activity and stability properties by amination and modification with glutaraldehyde*´´ and the second one ´´Amination of ficin extract to improve its immobilization on glyoxyl-agarose: Improved stability and activity versus casein´´

Abstract

Ficin extract immobilized on glyoxyl-agarose was chemically modified using carbodiimide and ethylenediamine (full or partial amination of the carboxylic groups of the enzyme) or glutaraldehyde (modifying primary amino groups) under different conditions. Aminated enzyme altered its activity versus casein and benzoyl-arginine-p-nitroanilide (BANA), e.g., the activity versus casein even slightly increased while versus BANA it decreased, being this more significant at pH 9 (the fully aminated biocatalyst increased the activity versus casein by 10% but it decreased more than 5 folds versus BANA), greatly altering the enzyme specificity. The amination improved the enzyme stability at pH 5 while stability was impoverished at pH 9. Glutaraldehyde treatment usually decreased the activity versus BANA while it improved the activity versus casein (by around a factor of 2 when using 1% glutaraldehyde). Enzyme thermostability was enhanced, mainly at pH 7. This permitted to have more linear casein hydrolysis courses at pH 7 and 66 °C compared to the unmodified enzyme. Amination followed by glutaraldehyde treatment drove to the almost full enzyme inactivation. Thus, these treatments may be considered interesting for preparing an improved biocatalyst of ficin, mainly in proteolytic applications, and may be considered (mainly the amination) a possibility to further improve enzyme immobilization.

Keywords: Chemical immobilization of immobilized enzymes, Enzyme amination, Enzyme crosslinking. Enzyme modulation, Enzyme stabilization, Hydrolysis of proteins.

Résumé

La ficine immobilisée sur le glyoxyl-agarose a été chimiquement modifiée sous déférentes condition par utilisation de carbodiimide et de l'ethylenediamine (provoquant une totale amination ou une amination partielle des groupements carboxyliques de l'enzyme). Ou par utilisation de glutaraldéhyde (modifiant les amines primaire). L'amination provoque une perte importante d'activité. L'activité est un peu améliorée en utilisant la caséine comme substrat mais elle diminue avec la BANA, la diminution la plus significative est observée á pH 9 avec 10% d'augmentation avec les caséines mais une diminution de 5 fois comparée à l'activité initiale avec le BANA. L'amination améliore la stabilité de l'enzyme a pH 5, mais cette stabilité est dégradée à pH 9. Le traitement avec le glutaraldéhyde provoque une diminution de l'activité avec le BANA mais il l'amélioré avec les caséines. La thermostabilite est aussi améliorée surtouts à pH 7, permettant ainsi d'avoir une meilleure hydrolyse des caséines et d'avoir des courbe l'hydrolyse linéaire à 66ºC et pH 7. L'amination suivie de traitement avec le glutaraldéhyde provoque une perte d'activité. Ce traitement (amination) peut être intéressant pour la préparation de la ficine immobilisée et améliorée en particulier dans l'hydrolyse des caséines et les applications protéolytiques et pour une amélioration de l'immobilisation des enzymes.

Mots clés: *Modification chimique des enzymes immobilisées, amination enzymatique, réticulation enzymatique. Modulation enzymatique, stabilisation enzymatique, hydrolyse des protéines.*

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Solid phase chemical modification of agarose glyoxyl-ficin: Improving activity and stability properties by amination and modification with glutaraldehyde

El-Hocine Siar[a,b,1], Sara Arana-Peña[a,1], Oveimar Barbosa[c], Mohammed Nasreddine Zidoune[b], Roberto Fernandez-Lafuente[a,*]

[a] Departamento de Biocatálisis, Instituto de Catálisis-CSIC, Campus UAM-CSIC, 28049 Madrid, Spain
[b] Equipe TEPA, Laboratoire LNTA, INATAA, Université des Frères Mentouri Constantine 1, 25000 Constantine, Algeria
[c] Departamento de Química, Facultad de Ciencias, Universidad del Tolima, 730006299 Ibagué, Colombia

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ABSTRACT

Ficin extract immobilized on glyoxyl-agarose was chemically modified using carbodiimide and ethylenediamine (full or partial amination of the carboxylic groups of the enzyme) or glutaraldehyde (modifying primary amino groups) under different conditions. Aminated enzyme altered its activity versus casein and benzoyl-arginine-p-nitroanilide (BANA), e.g., the activity versus casein even slightly increased while versus BANA it decreased, being this more significant at pH 9 (the fully aminated biocatalyst increased the activity versus casein by 10% but it decreased more than 5 folds versus BANA), greatly altering the enzyme specificity. The amination improved the enzyme stability at pH 5 while stability was impoverished at pH 9. Glutaraldehyde treatment usually decreased the activity versus BANA while it improved the activity versus casein (by around a factor of 2 when using 1% glutaraldehyde). Enzyme thermostability was enhanced, mainly at pH 7. This permitted to have more linear casein hydrolysis courses at pH 7 and 66 °C compared to the unmodified enzyme. Amination followed by glutaraldehyde treatment drove to the almost full enzyme inactivation. Thus, these treatments may be considered interesting for preparing an improved biocatalyst of ficin, mainly in proteolytic applications, and may be considered (mainly the amination) a possibility to further improve enzyme immobilization.

1. Introduction

Enzymes are very useful tools in the modern organic and food chemistries, because of their very high activity under moderate temperatures and pressure, and by their high specificity (modifying only its substrate) and selectivity (giving only one product) [1,2]. This is very important in green chemistry, because it reduces energy consumption, the use of solvents and the production of side-products, facilitating the downstream of the products [3,4]. However, enzymes have these optimal properties towards their physiological substrate and under physiological conditions. In many instances, to have a suitable biocatalyst, it is necessary to improve the enzyme features versus the target substrates and under industrial conditions. Fortunately, nowadays there are many different options to improve enzyme features, like microbiology (use of thermophilic organisms, metagenomics) [5], genetic tools (site-directed mutagenesis, directed evolution) [6,7], or physicochemical modifications (chemical modification or immobilization).

Enzyme chemical modification is a potent means to improve enzyme features [8–10]. The objectives of protein chemical modification may be multiple. The simplest one is to alter the physicochemical properties of the enzyme surface. For example, the modification of the enzyme surface may pursue the improvement of the enzyme solubility in organic media, thus improving their performance in anhydrous media [11,12]. In some instances, this chemical modification may enhance enzyme activity/stability, usually under specific conditions [13–15]. A second and more complex objective is to introduce intermolecular crosslinkings [16]. In the case of multimeric enzymes, the crosslinking between enzyme subunits may enhance enzyme stability by avoiding subunit dissociation [17]. In the case of monomeric enzymes, the enzyme structure will be rigidified by the intermolecular crosslinking, and that may enhance enzyme stability under any

* Corresponding author at: ICP-CSIC,C/ Marie Curie 2, Campus UAM-CSIC, Cantoblanco, 28049 Madrid, Spain.
E-mail addresses: oveimar@gmail.com (O. Barbosa), zidounem@yahoo.fr (M.N. Zidoune), rfl@icp.csic.es (R. Fernandez-Lafuente).
[1] Both authors have evenly contributed to this paper.

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distorting condition [16]. The crosslinking will have a higher stabilization effect if the crosslinker length fits the length between the crosslinker groups, decreasing if it is much longer because it will not fix the positions of the involved groups. Thus, crosslinking using a multifunctional polymer will be relatively easy [18], but the rigidification effects may be limited [19,20] while using a bifunctional reagent, the number of crosslinkings will be lower, but the rigidification effects will be high [21].

One of the problems of enzyme chemical modification is that the reaction control and purification steps may be tedious; moreover there is some risk of enzyme precipitation. This may be solved using immobilized enzymes [22–24]. Enzyme immobilization is a required step to have an easily reusable biocatalyst [25–29], and it is another tool to improve enzyme properties, like stability, activity, selectivity, specificity or purity [30–33]. Immobilization may be coupled to any other technique of enzyme stabilization [17,22,34,35], and in the case of protein chemical modification it will facilitate the handling of the enzyme, the control of the reaction and will prevent the enzyme precipitation.

In this paper, we will study the effects of two different chemical modifications on the properties of a protease extract, ficin, immobilized/stabilized on glyoxyl agarose [36] (Scheme 1). Ficin is extracted from the latex of fig tree, this enzyme extract is utilized in cheese production [37,38], meat tenderization [39,40], brewing [41] and pharmaceutical [42–44] industries. It has also been used in the production of antibodies via specific hydrolysis of some protein bonds, [45–47]. Ficin extract has four different isoforms, all of them have been crystallized and their structures are available [48,49].

The first performed modification has been the amination, using carbodiimide and ethylenediamine, of the exposed carboxylic groups of the enzymes [23,50,51]. This produces the alteration of the ionic interactions among the ionizable groups in the enzyme surface; these effects will be expected to be different depending on the pH value. Together with the direct effects on enzyme activity/stability, this modification may be a first step for some more ambitious goals, for example to increase the number of enzyme-support attachments during immobilization (covalent or ionic), [52–60], or to facilitate the introduction of chemical intermolecular [61] or intramolecular crosslinkings [21,62].

The second modification is the treatment with glutaraldehyde [63]. This will introduce a moderately hydrophobic group pending on the primary amino groups, but if there are some of these groups nearby, some intermolecular crosslinking may be achieved if incubated under proper conditions.

Finally, we will modify the previously aminated enzyme with glutaraldehyde, where a more intense crosslinking may be expected [21].

The effects of the modifications will be analyzed using two substrates, a small one, benzoyl-arginine-p-nitroanilide (BANA), and a large one, casein.

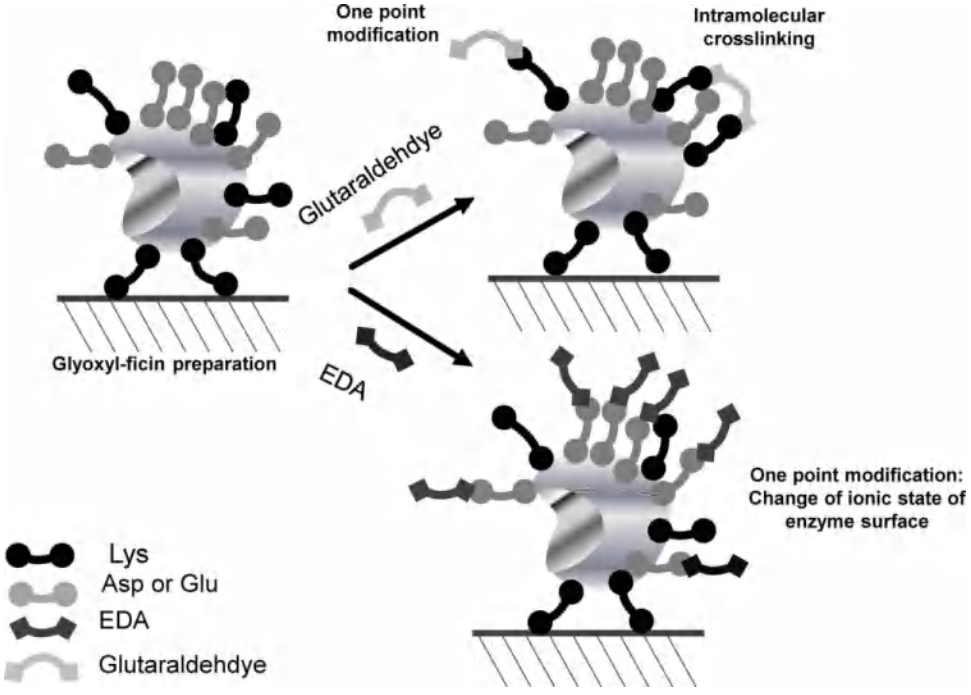
2. Materials and methods

2.1. Materials

Glycidol, glutaraldehyde (25% (v/v)), sodium borohydride, sodium periodate, ethylenediamine (EDA), 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), hydroxylamine, benzoyl-D,L-arginine-p-nitroanilide (BANA), cysteine and casein were purchased from Sigma-Aldrich (St. Louis, MO, USA). Agarose beads 4 BCL support was purchased from Agarose Bead Technologies (ABT), Spain. Glyoxyl agarose beads were prepared as previously described [66,67]. All other reagents were of analytical grade. All experiments were performed by triplicate and the reported values are the mean of the results of this set of experiments with their standard deviation.

2.2. Preparation of *fi*cin extract

Fresh fig latex was obtained from immature green fruits and leaves of Ficus carica L. growing in Kabylia, north of Algeria (Adekar, Bejaia). The latex was centrifuged at 3200 g for 15 min at 4 °C [68] to remove gums and other residues. The supernatant was utilized as the "crude extract of ficin" utilized in this paper (with a concentration of 98.5 mg protein/mL), stored at −20 °C until use. The protein concentration was quantified by Bradford's method [69]. A curve using BSA was used as standard.



Scheme 1. Different chemical treatments utilized to improve glyoxyl-ficin performance: schematic representation and likely effects.

2.3. Studies of enzyme structure and aminoacid solvent accessibility

Protein structures were visualized using PyMol software version 0.99rc6 [DeLano W. Pymol: An open-source molecular graphics tool. CCP4 Newsl Protein Crystallogr 2002]. Solvent Accessibility Prediction of residues of 4YYQ, 4YYR, 4YYV and 4YYW were calculated by the web-based program PROFacc, PredictProtein server, (www.predictprotein.org). PROFacc predicts solvent accessibility of protein residues for 10 states of relative accessibility. These are grouped into two states: buried and exposed [70].

2.4. Enzymatic assays

Activity of free and immobilized ficin extract was measured using BANA and casein as substrates.

For the standard method of enzyme activity determination using BANA was described by Santos et al. [71], 43.5 mg of BANA was dissolved in 1.0 mL of dimethyl sulfoxide and diluted to 100 mL using 0.1 M sodium phosphate pH 7, containing 5 mM EDTA, at 50 °C. The enzyme activity was calculated by measuring the p-nitroaniline formed at 405 nm (ε 8800 for p-nitroaniline under these conditions). The activity of ficin was expressed as the amount of p-nitroaniline (μmols) formed by 1 mg of protein per min.

Kunitz method to determine proteolytic activity was used with some modification [72]. 1 g of casein was diluted in 100 ml of 100 mM sodium phosphate at pH 7.0 containing 5 mM cysteine hydrochloride and 5 mM EDTA at 50 °C (in operational stability studies), 55 °C (in standard activity determination) or 66 °C (comparing immobilized and immobilized/modified enzyme prpearations). To 1 mL of substrate solution, 100 μL of ficin (free 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. These conditions produced the precipitation of the protein while the small peptides remain soluble. The absorbance of soluble peptides was measured at 280 nm. As reference, the substrate was added after the enzyme was first inactivated by incubation in TCA. One unit of activity is defined as the amount of enzyme that increases the absorbance by 0.001 min^{-1} under the given assay conditions.

2.5. Immobilization of *fi*cin extract

Immobilization of ficin on glyoxyl-agarose beads was performed as described in [36] by adding 10 g of glyoxyl agarose to 100 mL of 1–12 mg/mL ficin extract solution prepared in 50 mM sodium carbonate at pH 10.0 at room temperature under continuous stirring. After 3 h of enzyme support-reaction, 1 mg/ml of solid sodium borohydride was added and after 30 min, the immobilized enzyme was washed with distilled water.

2.6. Chemical modification of the *fi*cin extract immobilized on glyoxyl agarose beads

2.6.1. Amination of carboxylic groups

A mass of 8 g of ficin immobilized on glyoxyl agarose beads was suspended in 80 mL solution of 1 M EDA at pH 4.75 or pH 6. Solid EDC was added to the suspension to the desired concentration (1 mM or 10 mM) [14,21] or in 10 mM EDC at pH 6. After 90 min of gentle stirring at 25 °C, 100% modification of the exposed carboxylic groups was achieved using 10 mM EDC and pH 4.75, while 40–50% was modified using 1 mM EDC or 10 mM and pH 6 [14,21]. In some instances the modified ficin was filtered and 4 g were incubated for 24 h in a 0.1 M hydroxylamine solution at pH 7 to recover the modified tyrosines [65]. The final preparation was filtered and washed with 25 mM sodium phosphate at pH 7.5 and with an excess of distilled water.

2.6.2. Modification with glutaraldehyde

Eight grams of glyoxyl-ficin were suspended in 80 ml of 25 mM sodium phosphate at pH 7 containing 0.03%, 0.1% or 1% (v/v) of glutaraldehyde for 1 h. The biocatalyst was washed with distilled water and 1 g was suspended in 10 ml of 25 mM carbonate buffer pH 8.5 for 24 h. Activity was followed using the BANA assay during the modification. Then, 80 ml of water were added and sodium borohydride was added to reach a concentration of 1 mg of / ml, stirred for 30 min and washed with an excess of water.

2.7. Ficin biocatalyst stability assays

1 g of immobilized enzyme was suspended in 10 mL of 50 mM sodium acetate at pH 5, 50 mM sodium phosphate pH 7 or 50 mM sodium carbonate at pH 9 at 55 °C (or 56 °C using the glutaraldehyde modified enzyme). Samples were periodically withdrawn and the activity determined using the BANA assay described above.

3. Results

3.1. Immobilization of *fi*cin extract on glyoxyl-agarose using di*ff*erent enzyme loadings

Table 1 shows the immobilization yield and the expressed activity versus BANA and casein when using different enzyme loadings of glyoxyl-agarose beads. Maximum loading seems to be around 70 mg ficin/g of glyoxyl agarose. However, the most significant fact is the very dissimilar effect of enzyme loading on enzyme activity using BANA and casein. While with the small substrate the increase of the enzyme loading presented a moderate effect on enzyme expressed activity (from 60 to 45%) very likely caused by diffusional limitations, using casein the expressed activity rapidly decreased after 30 mg/g, and became less than 10% when overloading the support. This may be explained by the generation of steric problems to the entry of the substrate to the active center of the enzyme caused by nearby immobilized enzyme molecules [31,32], in case it is not fully exposed to the reaction medium, or by the higher diffusion limitation generated by the larger size of the casein. These results are similar to those obtained using glutaraldehyde as immobilization method [64]. A biocatalyst having just 10 mg of ficin extract per g of support was used in further studies. This was selected to reduce the diffusion limitations and permitted a fairer comparison [13–15,22–24,73]. Moreover, as it permits to have a more realistic activity versus casein, it will permit to better determinate the effect of the modification versus casein.

3.2. Analysis of the enzymes structures

Figs. 1 1S-4S show the PDB structures of the 4 isoforms of ficin molecules, with special emphasis to the terminal carboxy group, Asp and Glut, as well as that of the Lys and the terminal amino group.

From the figures, it looks that the lateral side of most isoforms of ficin (the left side of the active center in the figures) is the richest area of the proteins in Lys groups and therefore may be involved in the protease immobilization, this will fit with the good activity versus large

Table 1
Effect of loading on the expressed activity versus casein and BANA of ficin immobilized on glyoxyl-agarose. Experiments were performed as described in Methods section (casein, 55 °C).

Enzyme Loading	Immobilization Yield, (%)	Relative activity, (%), BANA	Relative activity, (%), Casein
10 mg/g	≥97	60 ± 6	55 ± 5
30 mg/g	90 ± 5	55 ± 6	48 ± 5
60 mg/g	77 ± 5	50 ± 4	17 ± 2
120 mg/g	55 ± 4	45 ± 5	8 ± 2

substrates using lowly loaded preparations while a low activity was obtained using a high loading, suggesting that the proximity of other enzyme molecules could hinder the accessibility of the enzyme active site to the large casein.

Regarding the chemical modifications, Figs. 1S–4S show that there are a large amount of carboxylic groups that can be modified using EDA and EDC, in many instances changing attractive interactions by repulsion interactions, in some instances changing repulsion ones by attractive ones (e.g, when a Lys is near to a Arg). Thus, an important change on the enzyme surface ionic interactions should be produced after amination.

Figures S1-4S show many Lys near enough to permit an intermolecular crosslinking by glutaraldehyde. However, glutaraldehyde treatment should affect amino groups not involved in the immobilization on the glyoxyl support. Thus, it is not easy to guess if some intramolecular crosslinking may be introduced. It should be considered that during immobilization some enzyme distortion may be produced, and this may greatly alter the exposition of the groups.

3.3. Chemical amination of glyoxyl *fi*cin

3.3.1. E*ff*ects on enzyme activity

The immobilized ficin was aminated under conditions where all exposed carboxylic groups should be modified, or where only around 40–50% of the exposed carboxylic groups will be aminated [21]. In one case, this lower modification is achieved by using a lower amount of EDC, that did not permit full protein modification. In the other case, the use of a higher pH value means that we modify the most reactive carboxylic acids, this pH also reduce the stability of EDC. As indicated in methods, after modification, part of the biocatalysts were incubated in hydroxylamine to regenerate any likely modified Tyr [65].

The first point that is clear in Table 2 is the very negative effect of the hydroxylamine incubation on enzyme activity. When a similar incubation was performed using the unmodified ficin preparation, its activity versus both substrates was almost fully destroyed, even with a higher intensity than using the modified preparations (activity becomes undetectable). Thus, the negative effect on enzyme activity was caused by a direct action of hydroxylamine on the enzyme, perhaps distorting its structure as a component of the medium, perhaps producing an undesired chemical modification on the enzyme (as it is a potent nucleophile).

Full amination of the enzyme had a negative effect for biocatalyst activity versus BANA at pH 5 (retaining more than half of the activity), moderate at pH 7 (around 75%) and suffering a drop at pH 9 (under 20%). These effects are quite different versus casein: at pH 9 activities even slightly increased compared to the unmodified preparation, and at pH 5 the biocatalyst retained more than 70% of the original activity. Partial modification of the biocatalyst obtained by decreasing the carbodiimide concentration or increasing the modification pH value, gave similar qualitative values to the full modification, but the decrease in activity versus BANA was lower at pH 5 and 9 (mainly using the enzyme modified at pH 9). With casein the modification using 1 mM carbodiimide was quite similar to the values observed using full modification (a moderate decrease of activity at pH 5 and 7, a moderate increase at pH 9). However, the biocatalyst modified at pH 6 suffered a significant decrease of activity at pH 5 versus casein (retaining less than 20%), and slightly decreasing the activity at pH 9. This suggested that the carboxylic groups modified under both conditions may differ even if the degree of modification is similar. At low EDC concentration, the most relevant fact to determine the modified groups is the reactivity of those groups, at pH 6 and 10 mM EDC is more relevant the stability of the modified group.

On the other hand, as it may be expected, the effect of the modification on the enzyme activity greatly depends on the pH value where the activity is analyzed (as this may alter the interactions between the ionic groups introduced on the enzyme). Moreover, a strong effect of the substrate utilized for determining the enzyme activity was also found, the modification caused more problems in hydrolysis of BANA (an anilide) than in the hydrolysis of the peptide bonds of casein. That way, the enzyme specificity has been altered by the chemical modification. Similar enzyme activity and specificity alterations by immobilized enzyme amination have been previously reported, mainly using lipases [13–15,22–24,73,74].

3.3.2. E*ff*ects on enzyme stability

The unmodified and the 6 modified immobilized ficin preparations were submitted to inactivation at different pH values (Fig. 1). The most obvious result was the lowest stability exhibited by all enzyme preparations incubated in hydroxylamine under all studied conditions. Thus, this treatment was not only very negative for enzyme activity, but also for enzyme stability. Therefore, this step should be discarded for this enzyme.

At pH 5, chemical amination produced a slight increase in enzyme stability without significant differences when comparing the enzyme modified under the 3 conditions. At this pH, the total ionization of the fully aminated enzyme will be very high, maximizing the repulsions between the amino groups, that may contribute to fix the position of the different groups. At pH 7, a slight decrease in ficin stability may be observed for the fully aminated, while the other two biocatalyst presented a very similar stability to that of the unmodified enzyme. At pH 9 the most stable preparation in the first moments was clearly the unmodified preparation, while the fully aminated enzyme was the least stable one. However, after 24 h both partially modified enzymes presented a higher residual activity than the unmodified enzyme.

From these results, it seems that it is possible to aminate ficin without generating serious negative effects on enzyme stability or activity, and even this amination may have some positive effects under certain conditions. There is not a clear trend of the expected results on

Table 2
Effect of the glyoxyl-ficin amination on relative enzyme activity versus BANA and versus casein at different pH values. Experiments have been performed as described in Methods section. The enzyme loading used was 10 mg ficin extract /g of support. Activity is given as relative activity, considering 100% the activity of the unmodified biocatalyst at the stated pH value (casein, 55 °C). Treatment 1: 1.5 h in 1 M EDA plus 10 mM carbodiimide at pH 4.75. Treatment 2: 1.5 h in 1 M EDA plus 1 mM carbodiimide at pH 4.75. Treatment 3: 1.5 h in 1 M EDA plus 10 mM carbodiimide at pH 6. Treatment 4. Incubation for 24 h in 1 M hydroxylamine at pH8.

RELATIVE ACTIVITY, (%)								
Treatment								
pH	Substrate	NONE	1	1 + 4	2	2 + 4	3	3 + 4
5	BANA	100	56.5 ± 2	24.3 ± 1	70.7 ± 2	11.2 ± 0.8	72.9 ± 2.1	18.6 ± 1.1
	CASEIN		72.7 ± 2.3	5.1 ± 0.3	73.9 ± 2.8	10.6 ± ,2	19.1 ± 0.5	0.0
7	BANA		76.9 ± 2.7	30.7 ± 1.3	83.1 ± 0.3	13.7 ± 0.5	80 ± 3	26.2 ± 2.2
	CASEIN		83.1 ± 3.2	48.2 ± 2	72.8 ± 3.3	43.5 ± 2.4	83.0 ± 2.2	30.0 ± 1.3
9	BANA		17.1 ± 2.1	16.9 ± 14	40.1 ± 2.1	20.2 ± 2.1	57.2 ± 1.6	3.3 ± 0.5
	CASEIN		113.2 ± 3.1	28.94 ± 2	111.5 ± 2.8	54.0 ± 2.7	91.2 ± 3.1	44.2 ± 2.5

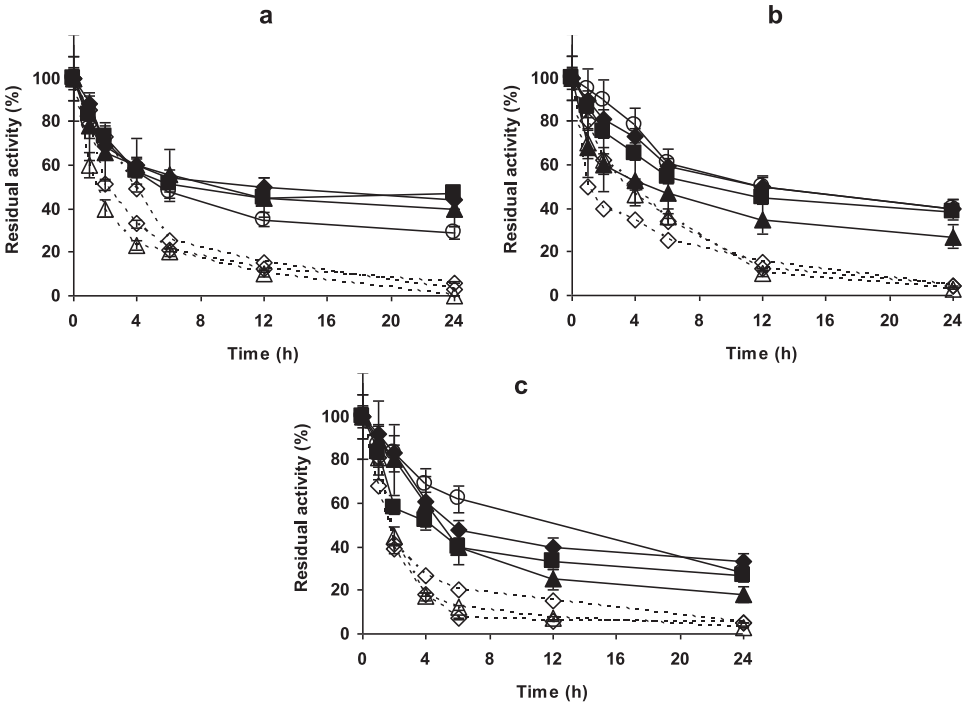


Fig. 1. Inactivation courses at different pH values (A: pH5, B:pH 7 and C: pH 9) and 55 °C of glyoxyl-ficin modified with EDA at different conditions. Other conditions are described in methods. Solid lines and solid symbols: enzymes modified with EDA and EDC, Dashed lines and empty symbols: enzymes modified with EDA and EDC incubated in 1 M hydroxylamine for 24 h at pH 8 as described in methods. Open circle: no modified; Triangles: enzyme modified with 10 mM EDC at pH 4.75; Squares: enzyme modified with 1 mM EDC at pH 6, Rhombus, enzyme modified with 10 mM EDC at pH 6.

enzyme stability of the amination [22–24]. In some instances, the amination of the enzymes has a high costs in terms of stability, like in the case of penicillin G acylase or glutaryl acylase [21,60], while in other cases, like some lipases, stability even increase at least under certain conditions [15,59]. That way, the amination of the enzyme may be used to improve enzyme immobilization [24].

3.4. Chemical modification of glyoxyl-ficin with glutaraldehyde

3.4.1. Effects on enzyme activity

Table 3 shows the effects on enzyme activity (versus BANA and casein) of modifying glyoxyl-ficin with different concentrations of glutaraldehyde for 1 h at pH 7. It should be remarked that the modification of this extract in aminated support with glutaraldehyde produced the almost full inactivation of the enzyme [64].

Here, although results were quite diverse and depended on the substrate and on the pH values, significat activities were recovered in all cases. Focusing on the activity with BANA, the modification with 0.03% glutaraldehyde produced no significant changes on enzyme activity at any of the studied pH values. Modification using 0.1% produced the same pattern of activity changes, but with a more significant decrease of activity at pH 5 or increase at pH 7 or 9 (by 20. When modified using 1% glutaraldehyde, the most relevant differences were the significant enhancement of the activity at pH 9 (166%); it is the only treatment that clearly enhanced the activity under these conditions, and also the decrease of activity at pH 7–55%.

Using casein, in all cases the activity increased when compared with the unmodified enzyme with scarce differences with the pH value or the glutaraldehyde concentration used in the modification (from 1.7 to 2.2 folds higher than the unmodified preparation).

Thus, this modification presented a clear positive effect on the proteolytic activity of glyoxyl-ficin, and changed the specificity of the enzyme. This may be caused by the changes on enzyme structure produced by the surface hydrophobization and the distortion likely generated by intramolecular crosslinkings.

The preparations were incubated for 24 h at pH 8.5 before reducing the composite with sodium borohydride (Fig. 2), showing some activity loss (a maximum of 30% when modified using 1% glutaraldehyde).

Table 3
Effect of the glyoxyl-ficin modification with glutaraldehyde on relative enzyme activity versus BANA and versus casein (55 °C) at different pH values. Experiments have been performed as described in Methods section using a biocatalyst having 10 mg of ficin extract/g of support. Results are given as relative activity, 100% is considered the activity of the unmodified biocatalyst at the designed pH value.

RELATIVE ACTIVITY, (%)					
Glutaraldehyde concentrations, (v/v) (%)					
pH	Substrate	NONE	0.03	0.1	1
5	BANA	100	94.5 ± 4.3	82.3 ± 3	78.5 ± 2.3
	CASEIN		200 ± 15	180 ± 17	171 ± 8
7	BANA		82 ± 3.7	120 ± 3.8	55.1 ± 2
	CASEIN		190 ± 20	204 ± 15	212 ± 12
9	BANA		78.3 ± 2.1	118 ± 3.4	166 ± 8
	CASEIN		191 ± 13	195 ± 15	188.5 ± 18

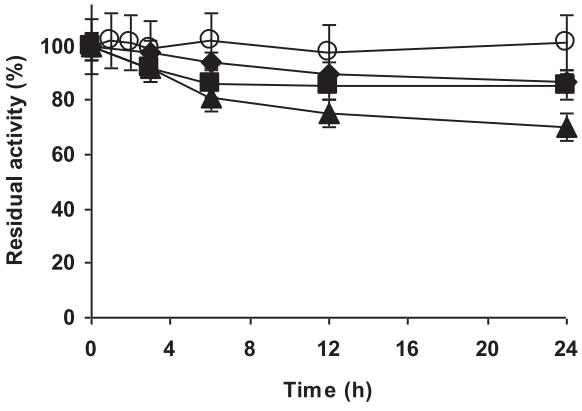


Fig. 2. Effect of the incubation at pH 8.5 and 25 °C of glutaraldehyde modified glyoxyl-ficin before sodium borohydride reduction. Experiments were performed as described in methods. Triangles: 1%, Squares, 0.1%, Rhombus: 0.03%, Open circles; unmodified glyoxyl-ficin.

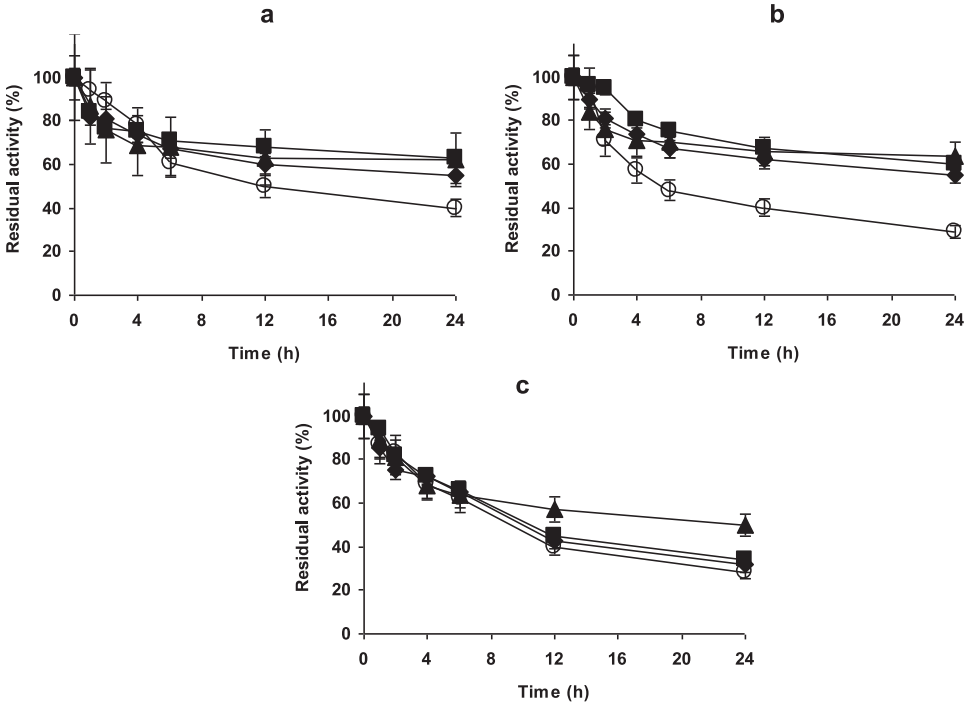


Fig. 3. Inactivation courses at different pH values (A: pH5, B: pH 7 and C: pH 9) and 56 °C of glyoxyl-ficin modified with different concentrations of glutaraldehyde. Other specifications are described in methods. Triangles: 1%, Squares, 0.1%, Rhombus: 0.03%, Open circles; unmodified glyoxyl-ficin.

These biocatalysts were used for further experiments.

3.4.2. E*ff*ects on enzyme stability

Fig. 3 shows the inactivation courses at different pH values of the 3 glutaraldehyde modified enzymes, prepared following the previous protocol, compared to the unmodified one. At pH 5, initially the unmodified biocatalyst retained higher activity, but after some time the modified preparations retained more activity, showing a hyperstabilized fraction. At pH 7, the higher stability of the modified preparations is evident since the first inactivation steps. All glutaraldehyde treated biocatalyst presented similar stability, although in general the treatment with 0.03% glutaraldehyde produced slightly lower stabilizations. This is more evident at pH 9, where the enzyme modified with 1% glutaraldehyde was the only one exhibiting a significant stabilization when compared to the unmodified enzyme.

The stabilization should be caused by the promotion of some intramolecular crosslinkings. A rapid look to Figs. 1S–4S shows that all isoforms of ficin have some Lys very near each other, where it is feasible to get an intramolecular crosslinking.

3.5. Glutaraldehyde modi*fi*cation of the aminated enzyme

Encouraged by the good results obtained in the individual modifications, the glutaraldehyde modification (using 1 and 0.1% glutaraldehyde) of all aminated glyoxyl-ficin preparations was performed. However, results were very different to those obtained modifying glyoxyl ficin with glutaraldehyde, even using casein as substrate, less than 5% of the enzyme activity was recovered in all cases. This suggested a very intense multi-crosslink of the surface of the aminated ficin with negative distorting effects on the enzyme structure, even using the partially aminated ones. Figs. 1S–4S clearly shows that the prospects of getting an intense crosslink are greatly increased after amination, apparently with very bad effects on enzyme activity. Therefore, the glutaraldehyde modification of aminated glyoxyl-ficin requires further optimization and studies to control it in order to keep a higher activity.

3.6. Hydrolysis of casein at high temperature

Thus, the most stable preparation produced in this paper was the glyoxyl-ficin modified with 1% glutaraldehyde. The hydrolysis of casein using glyoxyl-ficin and glyoxyl-ficin modified with glutaraldehyde at 66 °C was studied. It should be pointed that glyoxyl-ficin already exhibited a clear advantage compared to the free enzyme [36]. Fig. 4 shows that the glyoxyl-ficin worked for 2 h and then the activity became very low. However, using the glutaraldehyde modified enzyme, the enzyme can work for the 8 h we permitted the reaction to progress. This should be related to the stabilization achieved by the immobilization.

The immobilized and modified enzyme could be used at pH 7 and 50 °C for 5 consecutive casein hydrolysis cycles without any significant decrement on enzyme activity (results not shown).

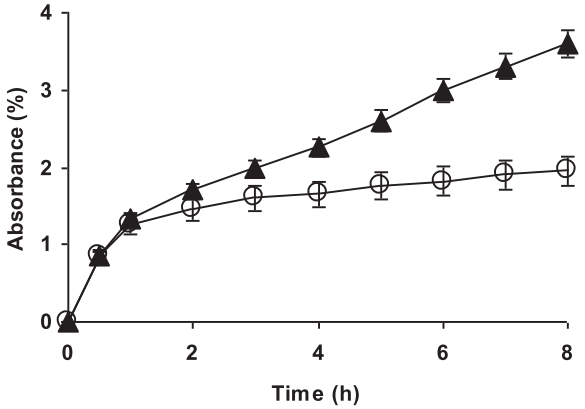


Fig. 4. Hydrolysis of casein by glyoxyl-ficin (open circles) or glyoxyl-ficin modified with 1% (v/v) glutaraldehyde (solid triangles) at pH 7 and 66 °C. Experiments were carried out as described in methods.

4. Conclusions

The chemical modification of glyoxyl-ficin may be an interesting tool to tune the enzyme features and as a first step in more complex strategies of biocatalyst design.

Amination decreased the activity versus BANA while it had a lower effect in the activity versus casein, the effect depending on the pH. Similarly, the amination altered enzyme stability: it is clearly improved at pH 5, at pH 7 results are unclear, while at pH 9 the effect is negative. This pH dependence on the effects of the amination may be expected considering that the pH will determine the ionization state of the new introduced amino groups. In any case, the results point out that it is possible to chemically aminate ficin, without any serious detrimental effect on enzyme features, for example, as a previous step to get a more intense multipoint covalent attachment during enzyme immobilization (using any support activated with groups directed towards primary amino groups) with the objective of enhancing enzyme stability, or even alter enzyme orientation to permit the use of fully loaded glyoxyl-supports in protein hydrolysis.

Glutaraldehyde treatment usually had a negative effect on enzyme activity versus BANA (decreasing to 55% using 1% glutaraldehyde), while doubled the activity versus casein. This was accompanied by a significant stabilization, mainly at pH 7 and 5. This could be caused by the promotion of some intermolecular chemical crosslinking, which enabled the rigidification of the enzyme structure [16]. This higher stability permitted to have more linear reaction courses in casein hydrolysis at pH 7 and 66 °C.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.procbio.2018.07.013.

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Conclusions

The chemical modification of glyoxyl-ficin may be an interesting tool to tune the enzyme features and as a first step in more complex strategies of biocatalyst design. Amination decreased the activity versus BANA while it had a lower effect in the activity versus casein, the effect depending on the pH. Similarly, the amination altered enzyme stability: it is clearly improved at pH 5, at pH 7 results are unclear, while at pH 9 the effect is negative. This pH dependence on the effects of the amination may be expected considering that the pH will determine the ionization state of the new introduced amino groups. In any case, the results point out that it is possible to chemically aminate ficin, without any serious detrimental effect on enzyme features, for example, as a previous step to get a more intense multipoint covalent attachment during enzyme immobilization (using any support activated with groups directed towards primary amino groups) with the objective of enhancing enzyme stability, or even alter enzyme orientation to permit the use of fully loaded glyoxyl supports in protein hydrolysis.

Glutaraldehyde treatment usually had a negative effect on enzyme activity versus BANA (decreasing to 55% using 1% glutaraldehyde), while doubled the activity versus casein. This was accompanied by a significant stabilization, mainly at pH 7 and 5. This could be caused by the promotion of some intermolecular chemical crosslinking, which enabled the rigidification of the enzyme structure [16]. This higher stability permitted to have more linear reaction courses in casein hydrolysis at pH 7 and 66 °C.

Conclusions

La modification chimique de la glyoxyl-ficine peut être un outil intéressant pour régler les caractéristiques de l'enzyme et comme première étape dans des stratégies plus complexes de conception de biocatalyseurs. L'amination a diminué l'activité par rapport au BANA alors qu'elle avait un effet plus faible sur l'activité par rapport à la caséine, cet effet dépend du pH. De même, l'amination altère la stabilité de l'enzyme: elle est nettement améliorée à pH 5, à pH 7 les résultats ne sont pas clairs, tandis qu'à pH 9 l'effet est négatif. Cette dépendance du pH vis-à-vis des effets de l'amination peut être attendue étant donné que le pH déterminera l'état d'ionisation des nouveaux amino groupes introduits. Dans tous les cas, les résultats montrent qu'il est possible d'aminer chimiquement la ficine, sans aucun effet néfaste sur les caractéristiques enzymatiques, par exemple, en tant qu'étape précédente pour obtenir une fixation par liaisons muliti-covalentes plus intense lors de l'immobilisation de l'enzyme (en utilisant n'importe quel support activé avec des groupes amino primaires) dans le but d'améliorer la stabilité de l'enzyme, ou même de modifier l'orientation de l'enzyme pour permettre l'utilisation de supports glyoxyl entièrement chargés dans l'hydrolyse des protéines.

Le traitement au glutaraldéhyde avait généralement un effet négatif sur l'activité enzymatique par rapport au BANA (diminuant à 55% en utilisant 1% de glutaraldéhyde), tout en doublant l'activité par rapport à la caséine. Ceci s'est accompagné d'une stabilisation significative, principalement à pH 7 et 5. Cela pourrait être causé par l'installation d'une certaine réticulation chimique intermoléculaire, qui a permis la rigidification de la structure enzymatique [16]. Cette stabilité élevée a permis d'avoir des courbes d'hydrolyse de la caséine plus linéaires à pH 7 et 66 ° C.

Abstract

Ficin extract has been aminated using ethylenediamine and carbodiimide to transform all exposed carboxylic groups into amino groups, retaining around 80% of activity versus benzoyl-D,L-arginine p-nitroanilide hydrochloride (BANA) and 90% versus casein. This aminated enzyme was then immobilized on glyoxyl agarose beads. After optimization of the immobilization protocol (immobilization at pH 10 for just 1 h), the new biocatalyst was compared to that obtained using the non-aminated enzyme. Activity versus BANA was lower, but was higher versus casein. The new biocatalyst was more stable than the reference mainly at pH 7. The new biocatalyst permitted to have a more linear course and a higher hydrolysis yield of casein at 75 °C. Moreover, the activity of the new preparations was significantly higher than the reference or the free enzyme in 8 M urea, at pH 7 and 55 °C. The enzyme in an overloaded biocatalyst exhibited a much higher specific activity versus casein (75% of the low loaded biocatalysts) than the non-aminated enzyme (only 30%), suggesting a more appropriate enzyme orientation that decreased steric hindrances. Finally, the enzyme was reused for 5 cycles of casein hydrolysis at 40 °C and pH 7 without any decrease in enzyme activity.

Keywords: Enzyme amination, Multipoint covalent attachment, Enzyme orientation, Casein hydrolysis, Steric hindrances.

Resumé

L'extrait de la ficine a été modifié en utilisant l'ethylenediamine et le carbodiimide pour transformer tous les groupements carboxyliques exposes à la surface en groupement amine. La ficine retiens environ 80% et 90% de son activité initial après transformation en utilisant le benzoyl-D,L-arginine p-nitroanilide hydrochloride (BANA) et les caséines respectivement comme substrat. La ficine obtenue après amination a été immobilisée sur le glyxyl-agerose. Apres optimisation de protocole d'immobilisation, le biocatalyseur obtenu est comparé avec celui obtenue avec la ficine non modifiée. L'activité a été dégradée par rapport au BANA mais elle améliorée par rapport aux caséines. Le nouveau biocatalyseur était plus stable que le biocatalyseur obtenu avec la ficine naturelle surtouts à pH 7. Ce biocatalyseur permet d'avoir un meilleur rendement d'hydrolyse des caséines à 75 ºC. En plus, ce biocatalyseur avait une activité significativement plus importante que celle de biocatalyseur obtenu avec le ficine naturelle ou la ficine libre en présence de 8 M de urea à pH 7 et 55 ºC. L'activité résiduelle après une charge maximale de support est plus importante avec le biocatalyseur obtenu après amination de ficine qu'avec celui obtenu avec la ficine naturelle (75 et 30% de l'activité initiale). Ce résultat montre que l'orientation de l'enzyme est améliorée après amination ce qui diminue les encombrements stériques. Le biocatalyseur obtenu avec la ficine aminée peut être réutilise dans 5 cycle d'hydrolyse des caséines sans perdre d'activité.

Mots clés: *amination des enzymes, liaisons multi-covalentes, orientation des enzymes, hydrolyse des caséines, encombrement stériques.*

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Amination of ficin extract to improve its immobilization on glyoxyl-agarose: Improved stability and activity versus casein

El-Hocine Siar [a,b,1], Roberto Morellon-Sterling [a,1], Mohammed Nasreddine Zidoune [b], Roberto Fernandez-Lafuente [a,*]

[a] Departamento de Biocatálisis, Instituto de Catálisis-CSIC, Campus UAM-CSIC, 28049 Madrid, Spain
[b] Equipe TEPA, LaboratoireLNTA, INATAA, Université des Frères Mentouri Constantine 1, 25000 Constantine, Algeria

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1. Introduction

Although enzyme properties make these biocatalysts very suitable candidates as industrial catalyst, their limitations (stability, selectivity, specificity, inhibition, reusability) are limiting their industrial implementation [1,2]. Among the many solutions [3–6], chemical modification of the enzymes [7–10] and their immobilization [11–14] have been described to be of great interest. Although in many instances they are considered secluded tools in biocatalysts design, this idea has been shown to be wrong over time. In fact, the combined use of chemical modification and immobilization of enzymes has been used in many instances to prepare significantly improved biocatalysts [15–19].

The chemical modification of previously immobilized enzymes permits to take the advantages of enzyme stabilization caused by the immobilization and those of solid phase modification [15–19]. Even in some cases, the immobilization protocol may determine the final effect of the modification on the enzyme properties [20–22].

Another possibility of coupling both strategies is to chemically modify the enzyme to improve its immobilization and obtain improved immobilized biocatalysts [18]. A clear example of this is to increase the number of nucleophiles on the protein surface to have more chances of getting an intense multipoint covalent attachment [17], which should produce higher enzyme stabilization [23]. Thus, in some instances, the enzymes have been aminated (chemically and in some instances genetically) [17] to enhance the properties of the immobilized biocatalysts. Using lipases, this modification used to be performed in a reversible immobilization form of the enzyme (e.g., via interfacial activation on hydrophobic supports) [24–26], to simplify the process, but that possibility is not viable using other enzymes [27,28]. Among the enzymes with industrial relevance, proteases have a special interest [29–34]. They have found applications mainly in food technology, but also in textile and fine chemistry or even biomedicine [35–39].

Compared to other enzymes, proteases have some peculiarities when used in immobilized form [40]. The first one refers to the size of the substrate when used in protein hydrolysis: only properly oriented enzyme molecules, exposing the active center to the medium, may be accessible to the substrate and therefore, will be active. The second one refers to the fact that proteases immobilized inside porous supports (but not in non-porous nano-materials) [41] are protected from the attack of other immobilized protease molecules, avoiding proteolysis.

* Corresponding author at: ICP-CSIC, C/ Marie Curie 2, Campus UAM-CSIC, Cantoblanco, 28049 Madrid, Spain.
E-mail address: rfl@icp.csic.es (R. Fernandez-Lafuente).
[1] Both authors have evenly contributed to this paper.

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Thus, immobilization of proteases to be used as industrial biocatalysts has special interest and problems [40].

Among the supports to immobilize and stabilize enzymes via multipoint covalent attachment, glyoxyl supports have proved to be very adequate to allow enzyme stabilization via multipoint covalent attachment [42]. The mechanism of enzyme immobilization on these supports is especially interesting: it requires the formation of several support-enzyme bonds to fix the enzyme to the support. That way, the enzyme is immobilized directly on the area where the highest possibilities of getting an intense multipoint covalent attachment exist [43]. Most proteins can be immobilized on glyoxyl supports only at very alkaline pH value, to permit the direct multipoint covalent immobilization. Exceptions to this general rule are the enzymes having several terminal amino groups in the same plane, due to the low K of these groups [44]. Thus, multimeric enzymes have been directly purified, immobilized and stabilized by immobilization on glyoxyl supports at neutral pH value [45]. In other cases, poly-Lys tagged proteins have been one step immobilized-purified [46]. Proteolyzed proteins, where several surface terminal amino groups are generated, may also be immobilized at lower pH values [47]. Most examples of coupling enzyme immobilization and chemical modification involve these activated supports. Chemical amination of the carboxylic groups of the proteins with ethylenediamine following the carbodiimide route permits the enrichment of the protein surface of amino groups [48,49], which have a lower pK than that of Lys (around 9.2) [50]. This greatly increases the protein reactivity versus the glyoxyl groups [28,51].

Moreover, the creation of new areas rich on amino groups may alter enzyme orientation, which is very important in the case of proteases [40]. In some cases, chemical amination has very negative effects on enzyme activity/stability [52]. In other cases (mainly involving lipases), amination produces a certain stabilization and/or increase in the activity, at least under certain conditions [53]. Among proteases, ficin is an interesting proteolytic extract obtained from the fig tree, containing different isoforms of proteases [54–58]. This proteolytic extract has different uses ranging from cheese making to proteolysis of proteins or synthesis of peptides [59–62]. Even recently it has been associated to a peroxidase activity [63]. Ficin extract has been previously immobilized on different supports [62,64–69]. One of the most stable immobilized biocatalysts was prepared using glyoxyl agarose, having a significant but not very high stabilization [68]. When the immobilized enzyme was aminated, its activity/stability remained almost unaltered [70]. Thus, it seems a good example to couple enzyme amination to enzyme immobilization. Interestingly, the enzyme immobilized on glyoxyl agarose retained a high percentage of activity versus casein when low enzyme loadings were used, but this value dramatically decreased using highly loaded enzyme preparations [70]. This suggested a relatively inappropriate enzyme orientation of the enzyme in the support, and that some immobilized enzyme molecules could block other enzyme molecules, reducing enzyme activity [70]. Here, we will aminate the protease ficin to immobilize it on glyoxyl agarose. The effects of the immobilization on the aminated enzyme activity/stability and also the effect of the biocatalyst loading on the activity of the enzyme versus casein will be investigated.

2. Materials and methods

2.1. Materials

Glycidol, sodium borohydride, sodium periodate, ethylenediamine (EDA), 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), benzoyl-D,L-arginine p-nitroanilide hydrochloride (BANA), cysteine and casein were purchased from Sigma-Aldrich (St. Louis, MO, USA). 4 BCL Agarose beads were purchased from Agarose Bead Technologies (ABT) (Madrid, Spain). Glyoxyl agarose beads were prepared as previously described [71,72] All other reagents were of analytical grade.

All experiments were performed by triplicate and the reported values are the mean of the results of this set of experiments with their standard deviation.

2.2. Preparation of *fi*cin extract

Fresh fig latex was obtained by crushing the immature leaves and green fruits of Ficus carica L. growing in Bejaia in the north of Algeria, and collecting the liquid in a clean flask at 4 °C. All the latex samples used in this study were collected freshly from the fig tree. The samples were centrifuged at 4000g at 4 °C for 15 min to remove resins and other debris. The insoluble material was discarded and the supernatant was used as "crude extract of ficin" (with a concentration of 79 mg protein/mL). This material was stored at −20 °C until use. The protein concentration was quantified by Bradford method [73] using BSA as the standard protein.

2.3. Enzymatic assays

Ficin activity was determined by two different protocols, using BANA or casein as substrates.

BANA was used following the method described by Devaraj et al. [74]. 43.5 mg of BANA were dissolved in 1.0 mL of dimethyl sulfoxide. Then, it was diluted to a total volume of 100 mL in 0.1 M sodium phosphate pH 7, containing 5 mM EDTA and 5 mM cysteine. The enzyme activity was determined by measuring the absorbance of the p-nitroaniline formed in this hydrolytic reaction at 405 nm (ε was 8800 for p-nitroaniline under these conditions). One unit of BANA activity was expressed as the amount of p-nitroaniline formed per 1 mg of enzyme per min.

Casein was used following the method described by Kunitz [75] with slight modifications. A solution of 1% (w/v) casein was prepared in 100 mM sodium phosphate at pH 7.0 containing 5 mM cysteine and 5 mM EDTA at 55 °C. To 1 mL of substrate solution, 200 μL 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 2 mL of 10% trichloroacetic acid (TCA), then it was incubated for 10 min at room temperature and centrifuged at 10,000 rpm. This treatment produces the precipitation of the protein, but the small peptides are maintained in solution. The absorbance of 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 casein activity is defined as the amount of enzyme that increases the absorbance by 0.001 min^{-1} under the given assay conditions.

2.4. Amination of the enzyme with EDC and EDA

The free ficin was added to 50 mL solution of 1 M EDA at pH 4.75. Solid EDC was added to the suspension to a final concentration of 10 mM. It has been reported that after 90 min of gentle stirring at 25 °C, 100% modification of all the exposed carboxylic groups was achieved [76]. The modified enzyme was then dialyzed at 4 °C for 24 h using a

Table 1
Effect of the amination on enzyme activity using BANA and casein as substrate.

	Activity (BANA)	% of residual activity (BANA)	Activity (BANA)	% of residual activity (BANA)
Ficin	1.31 ± 0.08	100 ± 0	1.085 ± 0,103	100.00
Aminated ficin	1.20 ± 0.10	94.61 ± 1.43	1.014 ± 0.079	93.43 ± 3.48
Aminated and dialyzed ficin	1.02 ± 0.12	81.17 ± 3.92	0.973 ± 0,101	89.67 ± 452

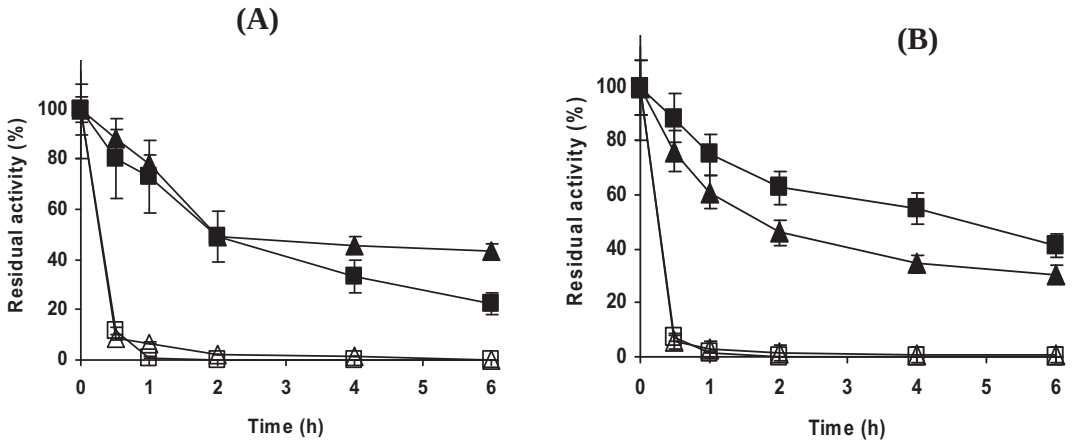

Fig. 1. Immobilization course of aminated and not aminated ficin in glyoxyl-agarose. Immobilization was performed at pH 9 (A) or pH 10 (B) and 25 °C using 10 g of support and 100 mL of 1 mg/mL ficin. Other specifications are described in Materials and methods section. Solid squares: suspension aminated ficin; empty squares: supernatant aminated ficin; solid triangles: suspension not aminated ficin:, empty triangles: supernatant not aminated ficin.

dialysis membrane with a molecular weight cutoff of 10 kD. Dialysis was carried out in 2.5 L of 5 mM phosphate buffer, the dialysis buffer was changed after 2 h, 4 h, 10 h and 24 h.

2.5. Immobilization of *fi*cin extract on glyoxyl agarose beads

In a standard experiment, immobilization of aminated or non-aminated ficin on glyoxyl-agarose was performed by adding 10 g of glyoxyl agarose to 100 mL of 1 mg/mL ficin solution prepared in 50 mM sodium carbonate at pH 9.0 or at pH 10.0 at room temperature under continuous stirring [68] In some cases, the enzyme immobilized at pH 9.0 was incubated after immobilization at pH 10.0.

To follow the immobilization courses, aliquots from suspensions and supernatants were withdrawn at different times and their catalytic activities were assayed. Free enzyme at the immobilization conditions maintained the activity unaltered during the whole immobilization process.

As a reaction end point, derivatives were reduced by addition of solid $NaBH_4$ to reach a concentration of 1 mg/mL, and incubated for 30 min [71]. The maximum immobilization time was 3 h (optimal value found using the not aminated enzyme) [68]. Then the derivatives were vacuum filtered and washed thoroughly with distilled water.

In some instances, the enzyme concentration was increased to increase the amount of offered enzyme (1, 3, 6 and 10 mg/mL were employed).

2.6. Determination of enzyme stability

The stabilities of the different ficin preparations were studied at pH 5 using sodium acetate buffer, pH 7 using sodium phosphate buffer or pH 10 using sodium carbonate buffer at 60 °C. Samples were periodically withdrawn and the activity was determined using BANA as substrate. Stabilizations were calculated from the ratio between the half-lives of the reference preparation and the problems biocatalysts, obtained from the inactivation courses.

2.7. Hydrolysis of casein

The hydrolysis of casein was performed as described by Siar et al. [68]. 1% (w/v) casein was prepared by dissolving solid casein in 0.1 M sodium phosphate at pH 7 containing 5 mM cysteine and 5 mM EDTA, samples of aminated and non-aminated ficin immobilized on glyoxyl-agarose were added in a thermostatized reactor under continuous magnetic stirring at 300 rpm. Hydrolyses were performed at pH 7 and at different temperatures (55 °C, 65 °C or 75 °C). In some cases, urea was added to the reaction mixture (until saturation) and the reaction was performed at 55 °C. Samples were periodically withdrawn and the absorbance after protein precipitation was determined at 280 nm as described above.

2.8. Reuse of the immobilized aminated an no aminated *fi*cin in the hydrolysis of casein

In order to test the possibility of the reuse of the immobilized enzyme in the hydrolysis of casein, five cycles of casein hydrolysis of 1 h were performed at 40 and 48 °C at pH 7 as described above. After 1 h of hydrolysis, the peptide production was checked and the immobilized enzyme was washed five times with 10 volumes of distilled water, and employed in a new reaction cycle of hydrolysis.

3. Results

3.1. Effect of the amination on enzyme activity

Table 1 shows that, in agreement with previous reports of the effect of amination of ficin immobilized in glyoxyl (that in that case produced a 25% decrease in BANA activity) [70], the amination of free ficin has an even lower effect on enzyme activity, the dialysis produced also a slight decrease on enzyme activity, perhaps due to protein autolysis. However,

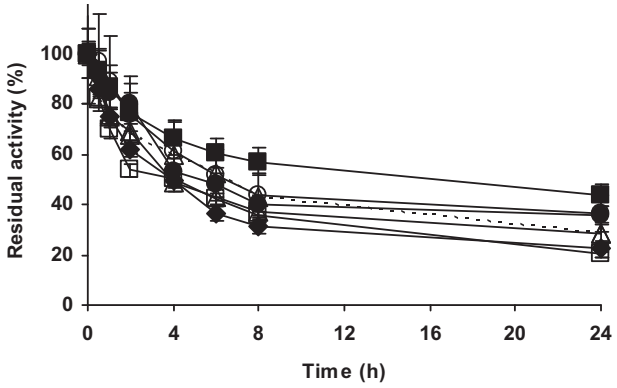

Fig. 2. Effect of immobilization time on the stability of ficin immobilized at pH 10. Inactivations were at pH 7 and 60 °C, using BANA as substrate. Other specifications may be found in Materials and methods section. 15 min (solid circles), 30 min (empty circles), 60 min (solid squares), 90 min (solid triangles), 120 min (empty squares), 180 min (empty triangles). Dashed line and empty triangles is the optimal preparation of the no aminated enzyme.

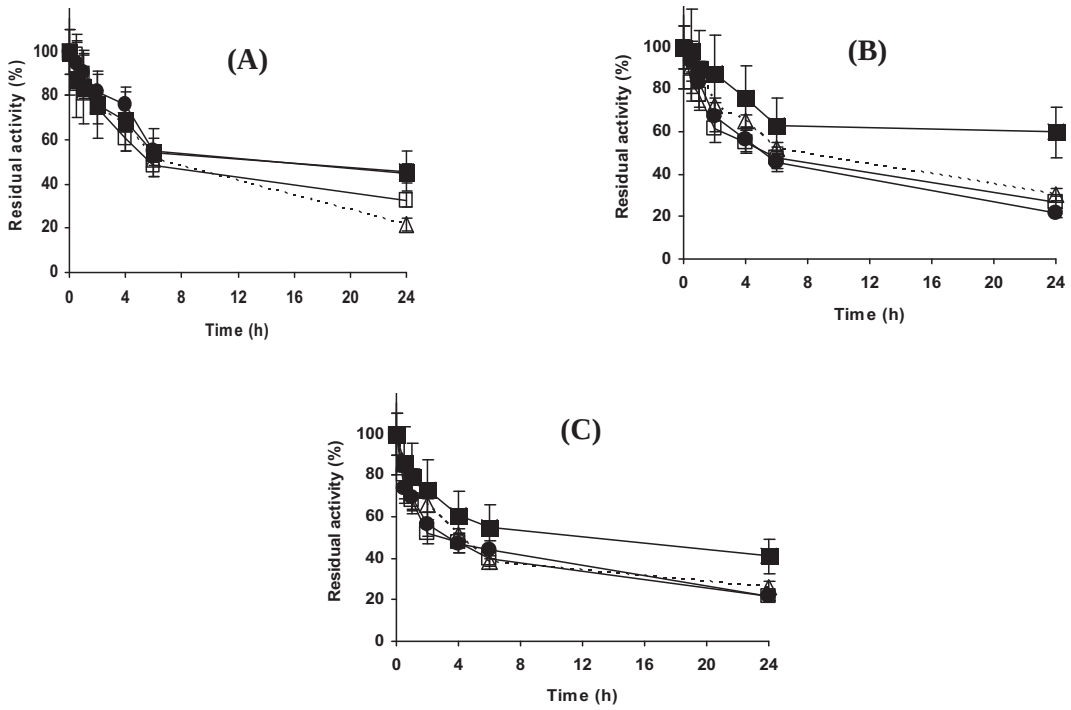

Fig. 3. Effect of the immobilization pH on the stability of aminated ficin immobilized on glyoxyl agarose compared to optimal ficin immobilized on glyoxyl agarose. Inactivations were performed at pH 5 (A), pH 7 (B) or pH 9 (C) and 55 °C. Inactivation was followed using BANA as substrate. Experiments were performed as described in Materials and methods section. Aminated ficin immobilized at pH 9 (solid circles), pH 10 (solid squares), pH 9 for 1 h and re-suspended at pH 10 for 1 h (empty squares), ficin immobilized on glyoxyl-agarose (empty triangles, dashed line). Experiments were carried out and at 55 °C.

while the amination of the immobilized enzyme produced an increase in the hydrolytic activity versus casein, using the free enzyme, the results were fairly similar to the BANA activity. This is a new example on how chemical modification may have different effects on enzyme properties, depending on the form (free, immobilized following different protocols, etc.) [21].

3.2. Immobilization of non-modified and aminated ficin on glyoxyl agarose

Fig. 1 shows the immobilization of aminated and non-aminated ficin on glyoxyl agarose at pH 9 and 10. Non-aminated ficin could be immobilized even at pH 9, very likely due to the existence of some lysis points that leave some extra amino terminal groups [71]. Immobilization is somehow faster using the aminated enzyme, although the differences are very short. Enzyme active decreased during the immobilization, in a stronger fashion using the aminated enzyme. At pH 10, immobilization for both ficin preparations is so rapid that it is not visible if the aminated enzyme is immobilized in a more rapid fashion than the non-aminated enzyme. However, Fig. 1B shows that in this instance the aminated enzyme preserved more activity after immobilization, although a more intense enzyme-support reaction may be expected.

3.3. Effect of incubation time on aminated and immobilized ficin stability

Using the enzyme immobilized at pH 10, the effect of immobilization time was studied on the final enzyme stability. The usual behavior on monomeric enzymes is an increase in enzyme stability when the enzyme-support reaction is more intense, as more enzyme-supports bonds are formed and the enzyme should become more stable [23,71]. However, ficin is an exception. Using glyoxyl, the optimal stability was found after 3 h [68], and then it started to decrease. Fig. 2 shows that using the aminated enzyme also follows a similar pattern, but in this case the highest stability was achieved when only 1 h of immobilization was allowed. The stability of the enzyme immobilized for only 15 or 30 min was slightly higher than that of the reference, after 1 h the stability was clearly higher than the reference and then started to decrease, even below the reference, and that occurred just after 90 min. This could be associated to a higher exposition of the catalytic Cys when the enzyme was distorted by the enzyme-support reaction. If oxidation of this Cys is relevant in enzyme inactivation, the fact that the polymer is more rigid did not compensate the more rapid oxidation of the Cys.

3.4. Effect of immobilization pH on the stability of the aminated enzyme

Three different biocatalysts of ficin were prepared, one immobilized at pH 10, another was immobilized at pH 9, and the last one was immobilized at pH 9 and then incubated at pH 10. In all cases, incubation time at pH 10 was fixed at 1 h. All were compared to the optimal non-aminated ficin glyoxyl biocatalyst. The study includes 3 different inactivation pH values, as the amination of the enzyme can have different effects on different pH values [17].

Table 2
Effect of immobilization pH on enzyme specific activity of aminated and not aminated ficin using casein and BANA as substrate. 100% is taken the as the specific activity of ficin immobilized on glyoxyl agarose beads. Experiments were performed as described in Materials and methods section.

	Activity casein	% residual activity casein	Activity BANA	% residual activity BANA
Aminated ficin immobilized at pH 9	1.454 ± 0.0231	134.49 ± 2.931	0.512 ± 0.098	60.81 ± 4.812
Aminated ficin immobilized at pH 10	1.293 ± 0.094	105.29 ± 6.624	0.736 ± 0.11	87.42 ± 3.514
Aminated ficin immobilized at pH 9 and then incubated at pH 10	0.970 ± 0.101	84.80 ± 4.518	0.504 ± 0.087	59.85 ± 5.718
Ficin biocatalyst	1.085 ± 0.093	100.00 ± 0	0.842 ± 0.125	100.000 ± 0

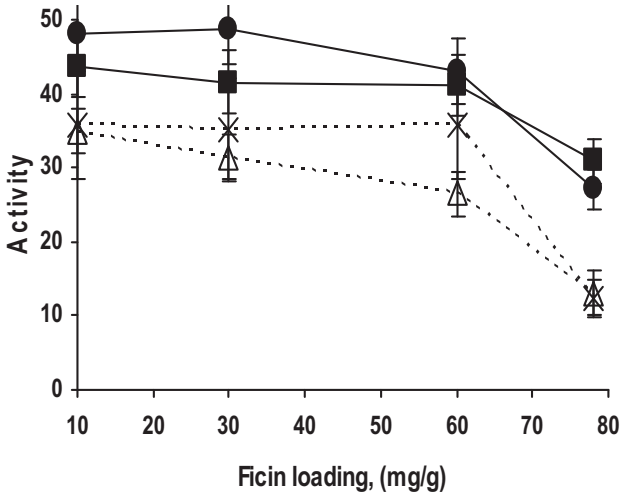

Fig. 4. Effect of enzyme loading on the activity of immobilized ficin versus casein. Experiments were carried out as described in Materials and methods section. Ficin immobilized on glyoxyl-agarose at pH 9 (empty squares, dashed line) or pH 10 (empty triangles, dashed line) and aminated ficin immobilized at pH 9 (solid circles) or pH 10 (solid squares).

Fig. 3 shows that the aminated enzyme immobilized at pH 10 was the most stable biocatalyst in all cases. At pH 5, all biocatalysts prepared using the aminated enzyme were more stable than the non-aminated enzyme. Incubation at pH 10 of the enzyme immobilized at pH 9 produced a decrease in enzyme stability, and the stability of the enzyme immobilized at pH 9 was very similar to that of the aminated enzyme immobilized at pH 10. When the inactivation was performed at pH 7, the situation was quite different. The enzyme immobilized at pH 10 was clearly the most stable one, and the two preparations of the aminated enzyme immobilized at pH 9 were slightly less stable than the reference, in this case the incubation at pH 10 gave a slightly higher stability. At pH 9 the only immobilized enzyme that was significantly different in terms of stability to the other ones, was the enzyme immobilized at pH 10, being the two biocatalysts immobilized at pH 9 very similar to the reference. Incubation for 30 min at pH 10 of the enzyme immobilized at pH 9 did not improve the results. That is, optimal stabilization was achieved if the aminated enzyme was immobilized at pH 10, and this difference was more significant in enzyme stability at pH 7. Results suggested that enzyme orientation when immobilized at pH 9 and pH 10 could be different.

3.5. Activity versus casein

Table 2 shows the activity versus casein and BANA of the different biocatalysts. Immobilized aminated enzyme was less active than the non-aminated enzyme versus BANA. Both biocatalyst prepared at pH 9 showed similar activities (around 60%), while the one immobilized at pH 10 preserved 80% of the activity (this means that considering the activity after amination, the immobilization at pH 10 has the same effect for aminated and not aminated enzyme). However, the enzyme activity increased versus casein for the enzyme immobilized at pH 9, although further incubation at pH 10 produced a significant decrease. If the enzyme was immobilized at pH 10, activity was slightly increased. Considering that the amination produced a 10% decrease in enzyme casein activity, these results pointed that the effects of the immobilization of the aminated enzyme on the activity versus casein were lower than using the non-aminated enzyme.

One of the main problems of the ficin immobilized on glyoxyl agarose was that this did not permit to fully load the support with ficin, as activity rapidly decreased when the loading increased, and this was attributed to the generation of steric hindrances between non-properly oriented and very nearby enzyme molecules [77]. It is possible that the use of aminated enzyme, where new areas of amino rich areas may be formed, can permit to alter this orientation. Fig. 4 shows the

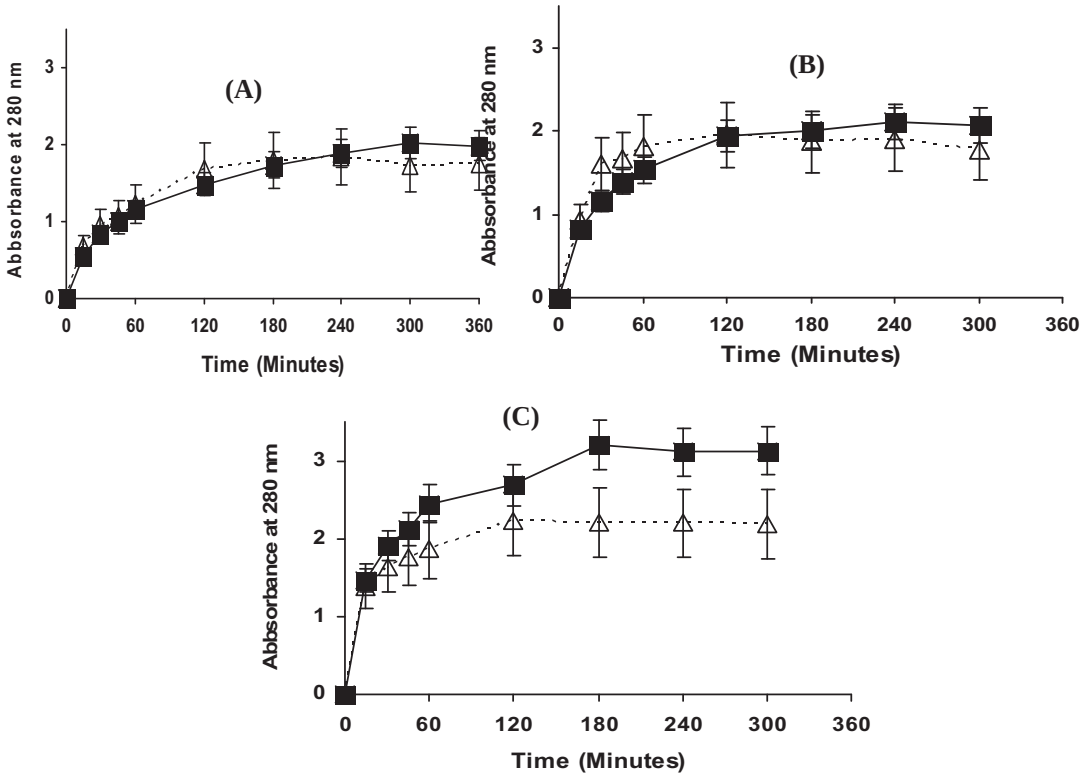

Fig. 5. Hydrolysis of casein at pH 7 and 55 °C (A), 65 °C (B) or 75 °C (C) by different ficin biocatalysts. Experiments were performed as described in Materials and methods section. Optimal biocatalyst of ficin (dashed line, empty triangles), optimal biocatalysts of aminated ficin immobilized on glyoxyl agarose (solid squares, solid line).

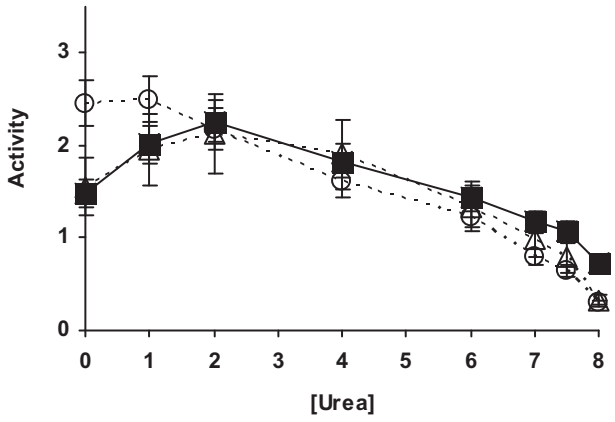

Fig. 6. Effect of urea in the courses of hydrolysis of casein by different ficin preparations. Reactions were performed at 55 °C and pH 7. Other specifications are described in Materials and methods section. Aminated ficin-glyoxyl (solid lines, solid squares), ficin-glyoxyl (dashed line, empty triangles) and free ficin (dashed line, circles).

effect of loading on the activity of ficin and aminated ficin derivatives prepared at pH 9 and 10. Ficin immobilized at both pH 9 and 10 showed a drastic drop in the recovered activity when immobilized at overload concentrations (activity is around 30% of the activity of non-fully loaded enzyme biocatalyst). This effect is only detected when going from 60 mg/g to 100 mg/g, and can hardly be explained by diffusion limitations. Using the aminated enzyme, at pH 9 the activity still is around 60% of the low loaded preparations when using overloaded enzyme preparations, and when immobilized at pH 10, 75% of the specific activity was still achieved using overloaded enzyme preparations. The enzyme molecules should be more rapidly immobilized at pH 10 than at pH 9, and also immobilization is more rapid for the aminated than for the non-aminated enzyme. This means that the nearest enzymes molecules should be those obtained for the immobilization of the aminated enzyme at pH 10, that are the ones with the highest specific activity. These results suggest that the enzyme orientation was better (active center was more exposed to the medium) for the aminated enzyme, mainly when immobilized at pH 10.

That is, together with a higher stability and a higher specific activity in casein hydrolysis, the aminated enzyme immobilized on glyoxyl agarose permitted to have a higher specific caseinolytic activity when using fully loaded enzyme biocatalysts.

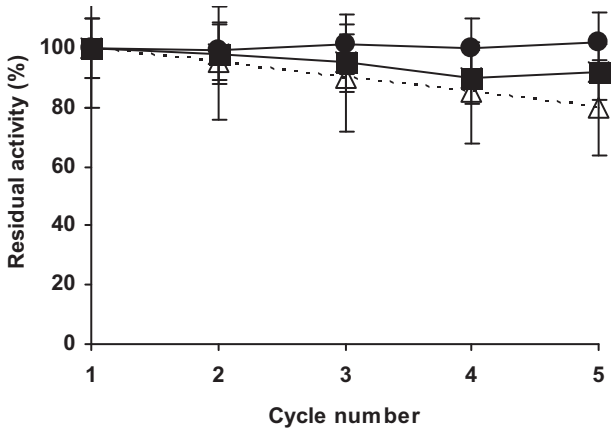

Fig. 7. Operational stability of optimal immobilized aminated ficin (solid line), at 40 (solid circles) and 48 °C (solid squares) and not aminated ficin (dashed line, circles) at 48 °C. Other specifications may be found in Materials and methods section. The hydrolysis in performed in the reactor at continuous agitation for one hour for each cycle at 48 °C (rapport ficin-glyoxyl/casein is 0.05 g/1 mL).

3.6. Hydrolysis of casein by optimal glyoxyl preparations of aminated and no aminated *fi*cin

One of the advantages of having more stable enzyme biocatalysts may be the possibility of using it under more drastic conditions. The aminated enzyme permitted a clear stabilization at pH 7, therefore we analyzed the biocatalysts performance at different temperatures. At the lowest assayed temperature (55°), the immobilized ficin exhibited a clear advantage compared to the free enzyme [68].When comparing the immobilized ficin with the aminated ficin biocatalyst, Fig. 5A shows that the reaction course using the new ficin biocatalysts may have a marginal advantage just in the last moments of the reaction. This advantage was similar at 65 °CFig. 5B, but becomes much clearer at 75 °CFig. 5C, where the new preparation permits to reach higher hydrolysis yields and give a more linear reaction course during the whole reaction.

We have also evaluated the activity of the biocatalysts in growing concentrations of urea. This chaotropic reagent may be utilized to redissolve enzyme aggregates, enabling their treatment, and can also indicate if the enzyme structure is really more stable [78,79]. As we have no information on the urea effect for the free enzyme, free ficin has been also studied.

Fig. 6 shows that using free ficin, 1 M urea marginally increases the activity, and at 2 M, it started to decrease. This increase in enzyme activity at 1 M may be just related to an easier hydrolysis of the casein, that will be less rigid. However, higher urea contents may affect the enzyme conformation leading to a lower activity. Ficin is quite resistant to urea even at 8 M (and 55 °C), >10% of the proteolytic capability was maintained. Using the immobilized ficin preparation, activity increased using 1 and 2 M urea, and then started to decrease, maintaining a 20% in 8 M urea. This suggested that the enzyme is more stable than the free enzyme, and for that reason the positive effects of urea now are observed even at 2 M urea, and also more activity is maintained at 8 M. The aminated enzyme enhanced the activity in a more significant way at 2 M of urea and retained 50% of the initial activity even in 8 M urea.

That way, the higher stability detected in inactivation experiments for the new biocatalyst could be correlated to a widening of the range of conditions where this new biocatalysts may be used.

3.7. Operational stability

One of the main objectives of immobilization is to reuse the biocatalyst for many cycles. Fig. 7 shows that the aminated enzyme biocatalysts could be reused for 5 cycles at 40 °C without any decrease in enzyme activity. We have also compared the operational stabilities of aminated and not aminated immobilized enzyme at 48 °C. In that case, the aminated biocatalysts lost <10% of activity after 5 cycles, while the non-aminated enzyme lost around 20%.

4. Conclusion

The chemical amination of ficin extract permitted to prepare an immobilized ficin biocatalyst with improved properties. The immobilization at pH 9 or pH 10 seems to involve different areas of the protein, as shown by the different activity/stability properties. An optimal immobilization time of just 1 h at pH 10 permitted to achieve the optimal activity/stability properties, longer immobilization times produces a decrease in enzyme activity and stability, perhaps by the exposition of the thiol group of the catalytic Cys to the medium. The new biocatalyst became more stable, more active versus casein and permitted the hydrolysis at high temperatures or in the presence of 8 M urea. However, the most relevant point may be the fact that it is now possible to overload the support with ficin without suffering a drastic reduction of protease activity, as it was found using the non-aminated enzyme. These results make these new ficin biocatalysts very promising ones.

Acknowledgments

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Conclusion

The chemical amination of ficin extract permitted to prepare an immobilized ficin biocatalyst with improved properties. The immobilization at pH 9 or pH 10 seems to involve different areas of the protein, as shown by the different activity/stability properties. An optimal immobilization time of just 1 h at pH 10 permitted to achieve the optimal activity/stability properties, longer immobilization times produces a decrease in enzyme activity and stability, perhaps by the exposition of the thiol group of the catalytic Cys to the medium. The new biocatalyst becamemore stable,more active versus casein and permitted the hydrolysis at high temperatures or in the presence of 8 M urea. However, the most relevant point may be the fact that it is now possible to overload the support with ficin without suffering a drastic reduction of protease activity, as it was found using the non-aminated enzyme. These results make these new ficin biocatalysts very promising ones.

Conclusion

L'amination chimique de l'extrait de ficine a permis de préparer un biocatalyseur de ficine immobilisée avec des propriétés améliorées. L'immobilisation à pH 9 ou pH 10 semble impliquer différentes zones de la protéine, comme le montrent les différentes propriétés d'activité / stabilité. Un temps d'immobilisation optimal de seulement 1 h à pH 10 a permis d'atteindre les propriétés optimales d'activité / stabilité, des temps d'immobilisation plus longs produisent une diminution de l'activité enzymatique et de la stabilité, peut-être par l'exposition du groupe thiol du Cys catalytique au milieu. Le nouveau biocatalyseur est plus stable, plus actif par rapport à la caséine et a permis l'hydrolyse à haute température ou en présence de 8 M. d'urée. Cependant, le point le plus pertinent peut être le fait qu'il est maintenant possible de surcharger le support avec de la ficine sans subir une réduction drastique de l'activité protéolytique, comme cela a été constaté en utilisant l'enzyme non aminée. Ces résultats rendent ces nouveaux biocatalyseurs de ficine très prometteurs.

Ficin is used traditionally in various applications such as production of bioactive peptides and fragments of antibodies via specific hydrolysis, pharmaceutical industries, meat tenderization, brewing industries and cheese making industries to produce the coagulation of the milk.

In this part of the work, we have used the immobilized ficin on glyoxyl agarose with various loading in milk caogulation in order to optimize the conditions for obtaining a better yield. this work is presented as a scientific paper with the title ´´ *Use of glyoxyl-agarose immobilized ficin extract in milk coagulation: Unexpected importance of the ficin loading on the biocatalysts*´´

Abstract

A protein extract obtained from fig tree (Ficus carica) latex containing ficin activity was immobilized on glyoxyl agarose. Different biocatalyst loadings were used (3, 10, 30 and 85mg/g). When casein was used as substrate, the expressed activities were 60%, 58%, 41% and 14%, respectively, very likely due to casein diffusional limitations. As expected, an increase of the concentration of either free or immobilized ficin reduced the clotting time of casein solution and milk. However, maintaining the same amount of ficin, lowly loaded ficin biocatalysts were unable to produce the clotting neither of the casein solutions nor of the milk, while highly loaded catalysts produced a good aggregate. Performing the proteolytic milk treatment at 4 °C to prevent aggregation and them incubating the milk at 40 °C, the use of immobilized enzyme in milk clotting gave coagulum yields of 19%, 24% and 27% for the 10mg/g, 30 mg/g and 85 mg/g immobilized ficin respectively, while free ficin gave a yield of around 20% under similar ficin concentrations.

Keywords: Ficin, Cheese, Modulation of enzyme functional properties by support loading, Milk clotting.

Resumé

L'extrait de la ficine a été immobilise sur le glyxyl-agaroseevec déférente charge finale de support en enzyme (3, 10, 30 et 85 mg/g), quand la caséine est utilisée comme substrat l'activité résiduelle est de 60%, 58%, 41% et 14 % respectivement, très probablement à cause des problèmes de diffusion des caséines dans le support. Comme prévu, l'augmentation de la charge enzymatique de la ficine libre ou immobilisée diminue le temps de coagulation des caséines et de lait. Mais, l'utilisation de la même quantité de la ficine immobilisée ne donne pas le même le résultat de coagulation, le support le moins charger sans incapable de coaguler les caséines ou le lait alors que les plus charge donne un bon résultat de coagulation. La coagulation a été réalisée à 4ºC pour éviter l'agrégation des caséines puis incuber a 40 ºC pour terminer la coagulation. Le rendement de coagulation était de 19%, 24% et 27% pour les biocatalyseurs charge avec 10, 30 et 85 mg/g, alors que pour le rendement avec l'enzyme libre était de 20%.

Mots clés: *Ficine, Fromage, Modulation des propriétés fonctionnelles enzymatiques par charge de support, Coagulation du lait*

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Use of glyoxyl-agarose immobilized ficin extract in milk coagulation: Unexpected importance of the ficin loading on the biocatalysts

El-Hocine Siar [a,b], Roberto Morellon-Sterling [a], Mohammed Nasreddine Zidoune [b], Roberto Fernandez-Lafuente [a,*]

[a] Departamento de Biocatálisis, Instituto de Catálisis-CSIC, Campus UAM-CSIC, 28049 Madrid, Spain
[b] Equipe TEPA, Laboratoire LNTA, INATAA, Université des Frères Mentouri Constantine 1, 25000 Constantine, Algeria

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A protein extract obtained from fig tree (Ficus carica) latex containing ficin activity was immobilized on glyoxyl agarose. Different biocatalyst loadings were used (3, 10, 30 and 85 mg/g). When casein was used as substrate, the expressed activities were 60%, 58%, 41% and 14%, respectively, very likely due to casein diffusional limitations. As expected, an increase of the concentration of either free or immobilized ficin reduced the clotting time of casein solution and milk. However, maintaining the same amount of ficin, lowly loaded ficin biocatalysts were unable to produce the clotting neither of the casein solutions nor of the milk, while highly loaded catalysts produced a good aggregate. Performing the proteolytic milk treatment at 4 °C to prevent aggregation and them incubating the milk at 40 °C, the use of immobilized enzyme in milk clotting gave coagulum yields of 19%, 24% and 27% for the 10 mg/g, 30 mg/g and 85 mg/g immobilized ficin respectively, while free ficin gave a yield of around 20% under similar ficin concentrations.

1. Introduction

Proteases have many different biotechnological uses, ranging from production of amino acids to synthesis of peptides [1–3], production of bioactive peptides or elimination of allergenic motifs [4,5], etc. Nevertheless, its main application is as a component of bioactive detergents [6–17]. Vegetable proteases have some advantages compared to mammalian ones, as the risk of disease transmission has reduced the interest of application of animal proteases. Among the vegetal proteases, ficin is an enzyme extract obtained from the sap of the fig (Ficus carica) composed by several proteases whose composition may be altered by the ambient conditions, health of the tree, watering, etc. [18,19]. Four components of ficin have been described and crystallized (A, B, C and D) and their structures are available [18,20]. All ficin components are glycoproteins with a high sequence similarity to bromelain [20]. The catalytic cysteine and a histidine are essential for the enzyme activity [18,21,22].

This enzyme extract is applied in many different areas. Modern applications include pharmaceutical industries [23], the production of bioactive peptides [24] and the production of fragments of antibodies via specific hydrolysis, as some researchers pointed that ficin is more rapid and consistent than other enzymes in the development of proteolytic hydrolysis maps of proteins [25,26]. The traditional uses include meat tenderization [27,28], brewing industries [29] and the one that will be the main topic of this paper, cheese making industries to produce the coagulation of the milk [30,31]. Milk clotting influences cheese yield, texture and flavor of the final product [32]. The traditional enzyme used in this reaction is calf rennet obtained from the fourth stomach of suckling calves. The limited production of this enzyme and the increase in cheese consumption makes convenient to look for calf rennet substitutes [33,34]. Recombinant rennets have been produced [35], but they are no permitted in some countries, leaving the vegetable proteases as good alternatives. However, these vegetable proteases have been reported to give in many instances bitter flavors [36]. Nevertheless, some interesting results have been obtained using different vegetable coagulants from different sources [37–44].

A good balance between proteolytic and clotting activities of the protein may become a critical point to be considered in the selection of an optimal milk clotting enzyme [36,45]. The rennet coagulation of milk starts with the proteolysis of the κ casein fragment of casein. This produces the breakage of the milk emulsion and initializes an enzyme-independent protein aggregation process. It has been reported that the clotting time is shorter when the rennet concentration is higher [46–49]. At first glance, the use of enzymes immobilized in porous supports as catalysts of this process may seem unappropriated, as the aggregates will close the pores of the support and to recover the solid catalysts from the solid coagulum may be nearly impossible, causing

* Corresponding author at: ICP-CSIC, C/Marie Curie 2, Campus UAM-CSIC, Cantoblanco, 28049 Madrid, Spain.
E-mail address: rfl@icp.csic.es (R. Fernandez-Lafuente).

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the reaction product and the immobilized enzyme to become part of the final product. However, hydrolyzed milk coagulation is strongly dependent on the temperature [50–57]. Thus, it has been showed that using immobilized rennet it is possible to perform the first step, the proteolysis at low temperatures (under 15 °C) and then to incubate the hydrolyzed milk at higher temperature to get the milk clotting. Since both processes are independent, it was possible to use different immobilized proteases in the first step [41,58–69].

One additional problem is that protease immobilization for this purpose must keep the enzyme active center properly oriented, otherwise, the large casein cannot accede to the active center (e.g., if it is oriented towards the support surface) [70,71].

In our laboratory, we have ficin immobilized and stabilized via multipoint covalent attachment on glyoxyl agarose beads [72]. Agarose may be considered a useful support for some applications [73]. With this immobilization protocol, enzyme preparations with very high caseinolytic activity were obtained [74]. It should remarked that the secondary amino bonds obtained after reduction with borohydride are extremely stable, able to stand even under acid hydrolysis of proteins [75–77].

However, the clotting of the milk with this not fully specific proteases presents a problem, as it is the possibility of a too intense hydrolysis of the milk proteins that finally can produce soluble peptides, with different (some time undesired) organoleptic properties.

Using immobilized enzymes in this process, one question appears: Is it equivalent to have the same amount of protease molecules using different enzyme loadings on the support? Initially, for complex processes like this, the answer should be negative, although we have not found any previous evidence on this regard in literature. Using immobilized enzymes, the concept of global enzyme concentration is no real, as all the enzyme is inside the biocatalyst particles and there is not enzyme in the other volumes of the reaction medium. That way, a protein that penetrates a porous biocatalyst with a low protease loading may suffer a milder modification than a protein that penetrates in a support with a very high protease loading.

Therefore, in this paper we have a double objective. First, to study the feasibility of using ficin extract immobilized in glyoxyl agarose to produce the milk clotting. Initially, a solution of casein will be used, and then skimmed milk will be utilized. Second, to check if the clotting activity of the immobilized ficin depends more on the total amount of protease than on the enzyme loading of the biocatalyst or vice versa, a question not raised in any other publication as far as we know.

2. Materials and methods

2.1. Materials

Glycidol, sodium periodate, sodium borohydride, cysteine, ethylenediamine, casein and benzoyl-D,L-arginine p-nitroanilide hydrochloride (BANA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 4 BCL Agarose beads were purchased from Agarose Bead Technologies (ABT), (Madrid, Spain). Glyoxyl agarose beads were prepared as previously described [2,76]. All other reagents were of analytical grade.

2.2. Methods

All experiments were at least performed by triplicate and results are supplied as the mean values and the standard error.

2.2.1. Preparation of *ficin* extract

Fresh fig sap was obtained by crushing the undeveloped leaves and green fruits of Ficus carica L. developing in Bejaia in the north of Algeria, and stored at 4 °C. The samples were centrifuged at 4000g at 4 °C for 15 min to remove solid debris. The insoluble material was discarded and the supernatant was used as "crude extract of ficin" (with a concentration of 73 mg protein/mL). This material was stored at −20 °C until use. The protein concentration was quantified by Bradford's method [79] using BSA as the standard protein, we use this value to refer the amount of ficin immobilized per g of support along the paper.

2.2.2. Amidase and protease activity assays

Ficin activity was quantified employing BANA or casein as substrates. BANA was used as described in [20]. A mass of 43.5 mg of BANA was dissolved in 1.0 mL of dimethyl sulfoxide. Then, it was diluted to a total volume of 100 mL in 0.1 M sodium phosphate at pH 7, containing 5 mM EDTA and 5 mM cysteine. In the assay, the increase in absorbance caused by the released p-nitroaniline at 405 nm was quantified (ε was 8800 for p-nitroaniline under these conditions). BANA activity was expressed as the micromols of p-nitroaniline formed per min per mg of ficin.

The proteolytic activity of the ficin preparation was measured using 1% (w/v) casein solution in 100 mM sodium phosphate at pH 7.0 containing 5 mM cysteine and 5 mM EDTA, following the method described by Kunitz [80] with slight modifications. 200 μL of ficin (enzyme extract solution or immobilized ficin suspension) were added to 1 mL of substrate solution at 55 °C. After 20 min, 2 mL of 10% trichloroacetic acid (TCA) were added to stop the reaction. The resulting suspension was incubated for 10 min at room temperature to permit protein precipitation and then centrifuged at 10,000 rpm. This treatment precipitated proteins, but small peptides remained in solution. The absorbance of supernatant was measured at 280 nm. As reference, substrate was added after the enzyme was inactivated by incubation in TCA. One unit of casein activity is defined as the increment of absorbance × 1000 per min^{-1} and per amount of enzyme under the given assay conditions.

2.2.3. Ficin immobilization

Immobilization of ficin on glyoxyl-agarose was performed as previously described [72]. Briefly, 10 g of glyoxyl agarose were added to 100 mL of ficin solution prepared in 50 mM sodium carbonate at pH 10.05, at room temperature under continuous stirring for 3 h [72], with an amount of protein enough for the different support loadings (from 0.3 to 10 mg/ml). As a reaction end point, derivatives were reduced by addition of solid $NaBH_4$ to reach a concentration of 1 mg/mL, and incubated for 30 min [76]. Then the derivatives were washed thoroughly with distilled water and vacuum filtered.

2.2.4. Casein clotting activity of *ficin* biocatalysts

The coagulant activity of immobilized and free ficin was determined following the method described by Pessela et al. [58] with slight modifications. A solution containing 1% (w/v) casein from bovine milk in 50 mM sodium acetate buffer and 5 mM sodium chloride at pH 5.6 was used as substrate. To start the reaction, the desired quantity of immobilized or free ficin was added to 2 mL of the substrate solution at 25 °C and the reaction was followed spectrophotometrically by the increment of absorbance at 550 nm promoted by the protein precipitation. Activity was inversely proportional to the inflection point of this curve.

2.2.5. Two step casein coagulation

The hydrolysis of 1% (w/v) casein was first performed at 4 °C to prevent the precipitation of the hydrolysate [58]. Samples were withdrawn periodically, heated at 40 °C for 20 min to permit the precipitation of the hydrolyzed protein. Then, the absorbance was determined at 550 nm.

2.2.6. Two step clotting of skimmed milk

The first step of the coagulation of skimmed milk (Celta, pH 6), the enzymatic one, was carried out at 4 °C for 2.5 h (the volume of milk used was 40 mL using 40 mg of ficin in immobilized form or 300 μg of free ficin). Then, when using immobilized enzymes, the samples were centrifuged to remove the biocatalyst taking advantage of the no precipitation of the casein hydrolysate at low temperature. In a second phase,

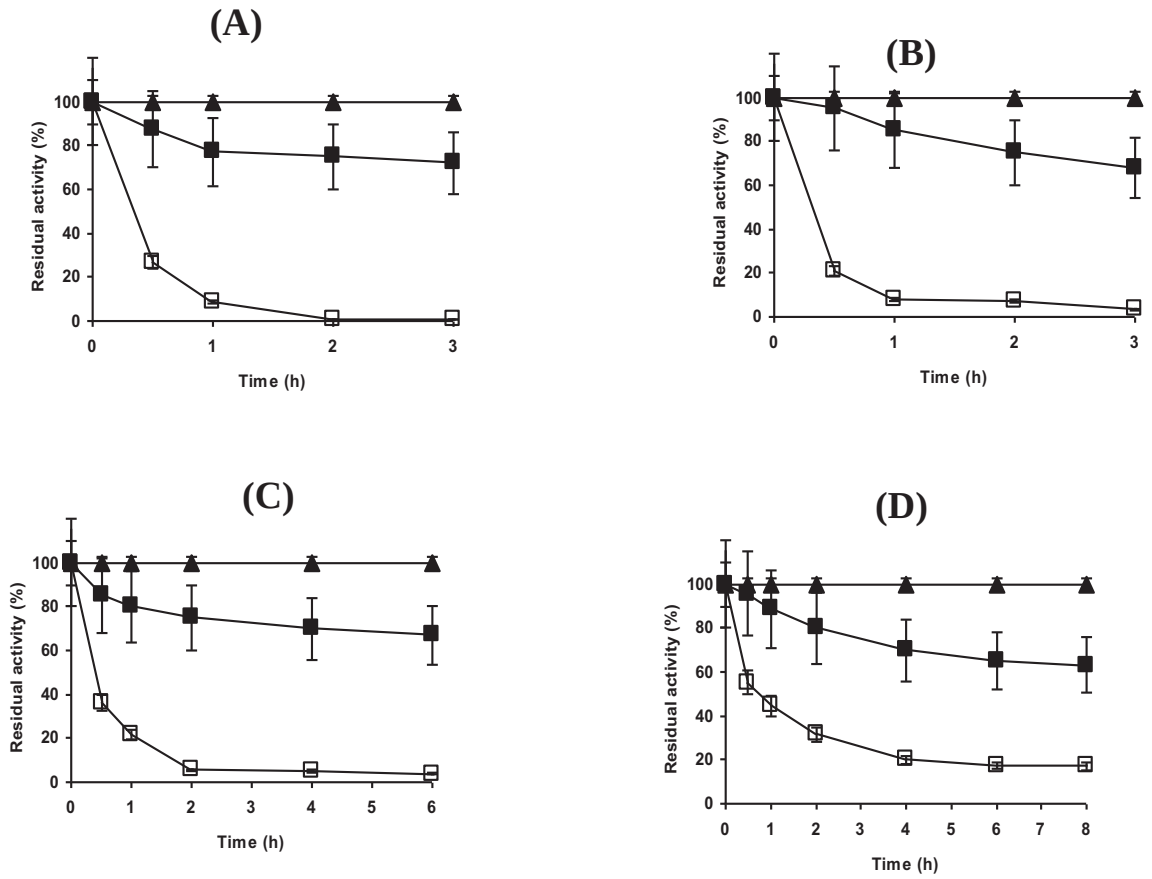


Fig. 1. Immobilization courses of ficin on glyoxyl-agarose beads. Immobilization was performed at pH 10 and 25 °C using 10 g of support and 100 mL of A: 3 mg ficin/g of support, B: 10 mg ficin/g of support, C: 30 mg ficin/g of support and D: 100 mg ficin/g of support. Other specifications are described in Materials and methods section. Solid squares: suspension; Empty squares: supernatant; Triangles: reference ficin solution incubated under identical conditions.

the supernatant was heated at 40 °C for 2 h (the time required to have a clear separation between coagulum and the whey) and coagulated casein was recovered by filtration (the filtration was carried out on a paper tissue for 15 min for each coagulum) and weighed. The yield was calculated according to the formula described by Jeantet et al. [81].

$$\text{Yield} = (\text{weight of recovered coagulum}/\text{weight of used milk}) \times 100.$$

2.2.7. SDS-PAGE analysis of the coagulum

SDS–PAGE was carried out according to LAEMMELI [82] using 12% separating and 4% stacking gels. The samples were mixed at a ratio of 1 mg of coagulum per ml of the buffer sample at pH 6.8 containing: 0.378 g TRIS base, 1 g of SDS, 2.5 mL of β-mercaptoethanol and 5 mL of glycerin. The volume was adjusted with H_2O until 25 mL.

Twenty micrograms of proteins were loaded on the gel and then subjected to separation in a running buffer containing 25 mM of Tris, 192 mM glycine and 0,1% SDS at a pH of 8,3 for 2 h at 100 V. After separation, the gel was stained for 1 h with a staining solution containing 3 mM Coomassie Brilliant Blue R-250 in 40% (v/v) ethanol, and 10% (v/v) acetic acid. Protein patterns were visualized after de-staining the gel in 40% (v/v) ethanol, and 10% (v/v) acetic acid until a clear background was achieved. A mixture of protein molecular weight markers obtained from Bio-Rad was used.

3. Results

3.1. Ficin immobilization

Fig. 1 shows the immobilization courses using 3, 10, 30 or 100 mg of ficin extract per g of support. Using enzyme loadings of 3 and 10 mg/g, the immobilization is very rapid (1 h is enough to immobilize all ficin activity) and expressed activity versus BANA was over 70% using the lowest loading and just under 70% using the highest load. To immobilize 30 mg/g, 2 h are required to fully immobilize the enzyme and expressed activity versus BANA decreased to just over 65%. This small higher decrease in expressed activity may be caused by an expected increase of the effects of the substrate diffusion limitations when the immobilized enzyme activity increases [83,84]. Using 100 mg/g, even after 8 h still some enzyme is not immobilized (because it is used more enzyme that can be immobilized on this support) [74] and expressed activity of the immobilized ficin was just over 60% (around 85 mg of ficin/g of support are immobilized). We used this time to ensure the full coating of the support surface with the protease molecules.

Table 1 shows the activity of the 4 immobilized biocatalysts versus casein. Biocatalysts mass activity increased with enzyme loading, but the specific activity of ficin decreased from 0.17 (using the biocatalyst with 3 mg/g) to less than 0.05 U/mg of ficin (using the immobilized preparation with 85 mg/g). The activity recovery moved from 60% to

Table 1
Activity of differently loaded immobilized ficin biocatalysts derivatives using casein as substrate. Experiments were carried out as described in Materials and methods section. Recovered activity was calculated by dividing the observed activity by the activity expected from the specific activity of the free enzyme.

Loading of the ficin biocatalysts	Caseinolytic activity, (U/g)	Recovered activity (%)
3 mg/g	0.51 ± 0.03	60 ± 3
10 mg/G	1.64 ± 0.12	58 ± 1
30 mg/g	3.52 ± 0.23	41 ± 2
85 mg/g	4.18 ± 0.32	16 ± 2

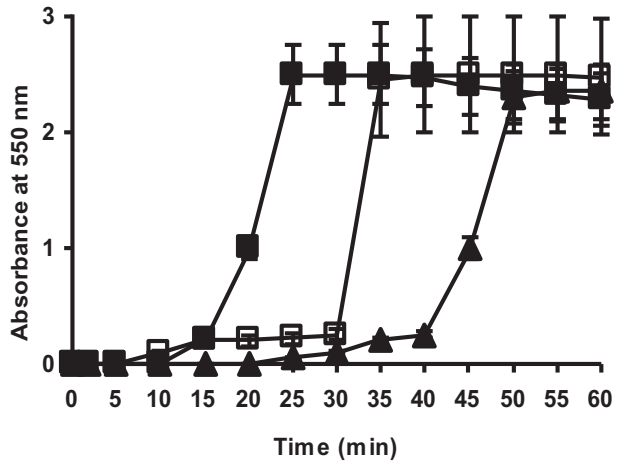


Fig. 2. Effect of ficin concentration on the clotting course. The Figure shows the evolution of the absorbance at 550 nm during the hydrolysis of casein solution (10 mg/ml) at 25 °C and pH 5.6. Solid squares: 15 μg of ficin/ml, Empty squares: 10 μg ficin/ml and Triangles: 5 μg ficin/ml. Experiments were carried out as described in Materials and methods section.

16%. This drop in activity using the fully loaded catalyst compared to the results using BANA was very likely due to the high diffusion limitations of the large casein and by the steric hindrances generated to recognize this large substrate using fully loaded biocatalysts, where one immobilized enzyme molecule can block other [74,85,86].

3.2. Coagulation activity of the different *ficin* preparations

Fig. 2 shows the increment of absorbance caused by the clotting of casein (a solution of 10 mg/ml) promoted by the ficin treatment using free enzyme using different ficin concentrations. The ficin concentration had a great effect on the clotting of the casein, as it has been previously described [46–49]: aggregation was more rapid using more concentration of ficin. It is remarkable that after some time, absorbance started to decrease, suggesting that the ficin is able to hydrolyze the components of the aggregates, producing some soluble peptides and reducing the percentage of aggregated protein. This is a negative fact, as it decreases the yield of coagulum.

The situation was fully different using the immobilized ficin. Fig. 3 shows the clotting of casein solution (10 mg/ml) using biocatalysts with different loadings and different final concentration of ficin in the assay, although identical for the biocatalysts with different loadings. That is, for each ficin concentration it was used the immobilized biocatalysts that gave the desired amount of enzyme, e.g., using the 10 mg/g biocatalysts, we used 3 times more amount than when using the 30 mg/ml. The least loaded biocatalyst (3 mg/g) was unable to produce casein clotting under any condition, even when the ficin concentration was highly increased (it has not been included in the figure). The biocatalysts having 10 mg ficin/g is far less active in the casein coagulation (per immobilized enzyme molecule) than the biocatalysts with 30 or 85 mg ficin/g, and also the preparation having 85 mg/g was more active than the biocatalysts with a load of 30 mg/g. It should be remarked that in all cases the same amount of enzyme (not of solid, but of immobilized enzyme) was employed, and that the caseinolytic ficin specific activity decreased when the loading increased (see

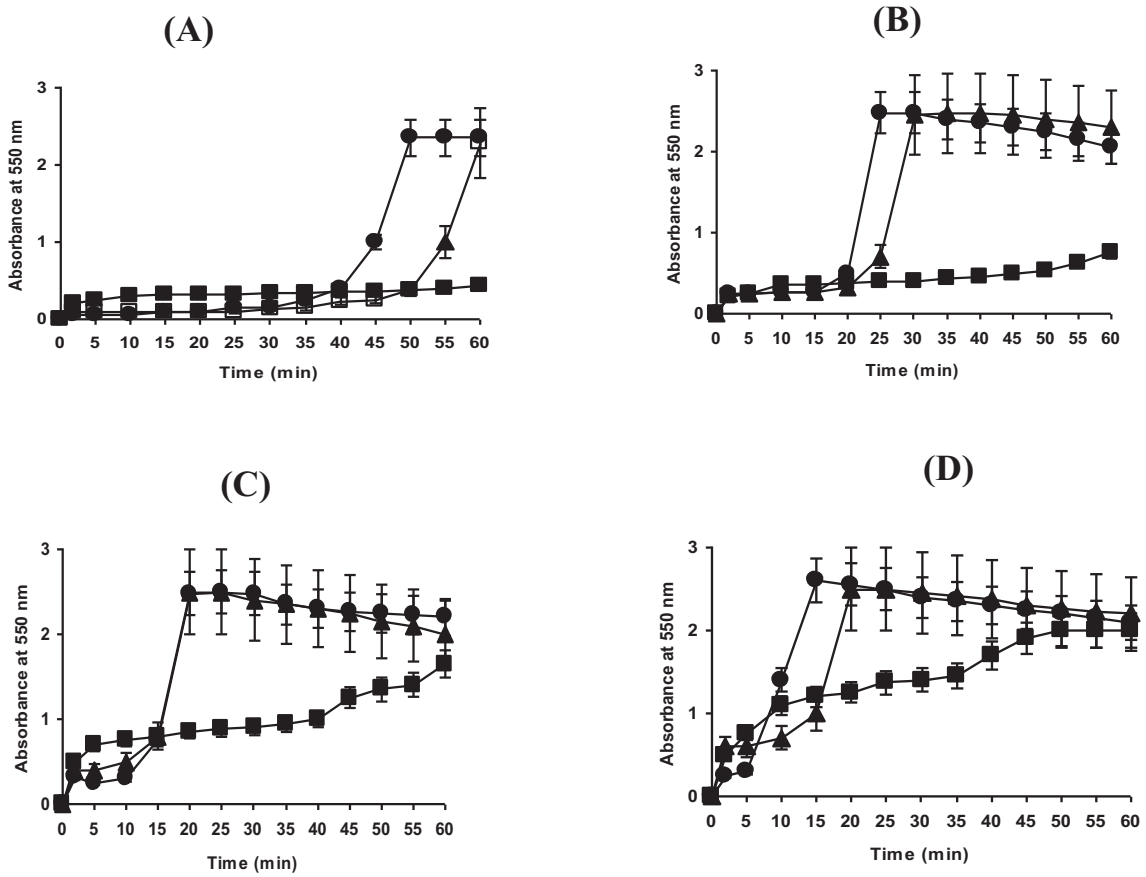


Fig. 3. Casein hydrolysis using immobilized ficin at different enzyme loadings. The Figure shows the evolution of the absorbance at 550 nm during the hydrolysis of casein (10 mg/ml) catalyzed by the different biocatalysts prepared in this paper, using different final global concentration of ficin. A: 0.25 mg ficin/ml, B: 0.5 mg ficin/ml, C: 1 mg ficin/ml and D: 1.5 mg ficin/ml. Solid circles: 85 mg ficin/g of support, Solid triangles: 30 mg ficin/g of support and Solid squares: 10 mg ficin/g of support) at 25 °C and pH 5.6. Experiments were carried out as described in Materials and methods section.

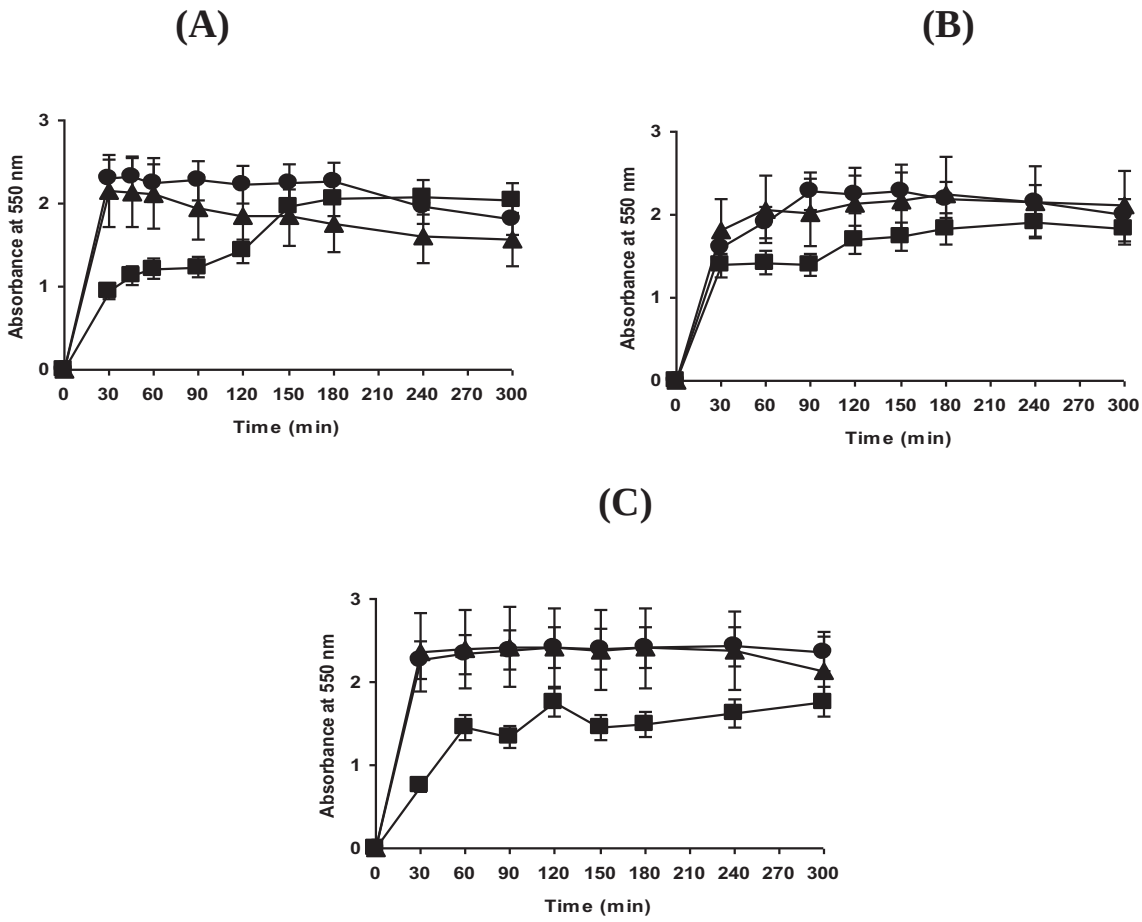


Fig. 4. Two-step clotting of casein by proteolysis at 4 °C and incubation at 40 °C using different ficin preparations. Circles: 85 mg/mg, Triangles: 30 mg/g and Squares: 10 mg/g. Experiments were carried out as described in Materials and methods section. A: 4 g of 10 mg/g immobilized ficin, 0.44 g of 30 mg/g immobilized ficin and 0.133 g of 85 mg/g immobilized ficin, suspended in 30 ml casein (10 mg/ml). B: 6 g of 10 mg/g immobilized ficin, 0.22 g of 30 mg/g biocatalyst and 0.0667 g of 85 mg/g biocatalyst, suspended in 30 ml casein (10 mg/ml). C: 2 g of 10 mg/g ficin biocatalyst, 0.15 g of 30 mg/g immobilized ficin and 0.0227 g of 85 mg/g ficin, suspended in 30 ml casein (10 mg/ml).

Table 1). As using free enzyme, a maximum in absorbance was reached and then it started to decrease, suggesting that the immobilized ficin was also able to hydrolyze some of the components of the aggregates.

That is, the clotting activity of the immobilized ficin biocatalysts followed a loading/activity dependence fully different to the caseinolytic activity, suggesting that in this more complex process, the most important parameter is not the global ficin "concentration", but the ficin loading of the biocatalysts. Using the preparation having 3 mg of ficin per gram of support, clotting was never observed even using 1 g of catalysts and 1 ml of casein solution. However, the highest loaded ficin preparation, with a much lower specific caseinolytic activity of the enzyme (see Table 1), showed a much higher coagulation activity. The difference is that a casein molecule that go inside the highly loaded biocatalysts finds a huge "superficial" concentration of ficin (ficin is just in the surface of the support pores), and that did not occur using the same amount of ficin molecules but in a less loaded biocatalyst. The exact mechanism of this phenomenon is out of the scope of this paper and can deserve some further investigation.

Table 2
Coagulation yields of skimmed milk submitted to casein hydrolysis using different ficin immobilized preparations. First phase of coagulation was performed at 4 °C and the second one at 40 °C. The experiment was performed at pH 6 using 40 ml of milk and 40 mg of ficin in immobilized form or 0.3 mg using the free enzyme. Other specifications are described in Materials and methods section.

Ficin preparation	Clotting yield %
Free enzyme	20.0 ± 1.5
Immobilized (10 mg/g)	19.2 ± 1.5
Immobilized (30 mg/g)	23.7 ± 1.8
Immobilized (100 mg/g)	27.4 ± 1.0

3.3. Two step coagulation of casein solutions using immobilized *ficin* biocatalyst

Fig. 4 shows the two step coagulation of casein solutions. For this purpose, casein solution was incubated with the biocatalysts at 4 °C using different concentrations of ficin. Under these conditions, casein aggregation was not appreciated during the experiments. Then, samples of the supernatant were taken at different time intervals, and incubated at 40 °C to favor the casein hydrolysate aggregation. Under these conditions, casein hydrolysate really aggregated and was possible to follow the increase in absorbance (caused by the casein coagulum) at different times of casein hydrolysis. The higher the concentration of casein (A, B o C), the faster the casein coagulum was obtained, in agreement with Fig. 3. Moreover, very clear differences were maintained between the biocatalysts with different loadings, even if the total amount of ficin was maintained in each experiment panel. That way, the 10 mg/g immobilized ficin biocatalyst remained as the least efficient biocatalyst to produce casein aggregates, but the differences with the other biocatalysts were shorter than when the hydrolysis was performed at 25 °C (Fig. 3). And differences between biocatalysts loaded with 30 or 85 mg/g were very negligible, while at 25 °C (Fig. 3) they were quite clear.

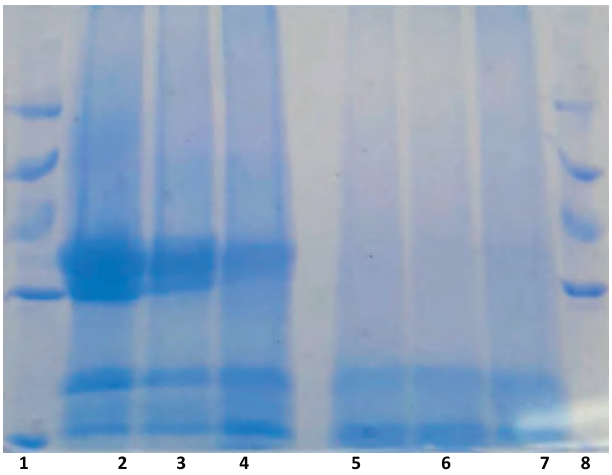


Fig. 5. SDS-PAGE study of the different obtained coagula. The total amounts of ficin used for the coagulation were 40 mg of enzyme/40 mL of milk for the immobilized ficin and 300 μg/40 mL of milk for the free ficin (hydrolysis time 2.5 h). Lanes 1 and 8: Molecular weight marker proteins, Lane 2: 10 mg/g immobilized ficin, Lane 3: 30 mg/g immobilized ficin, Lane 4: 85 mg/g immobilized ficin, Lanes 5–7: free ficin.

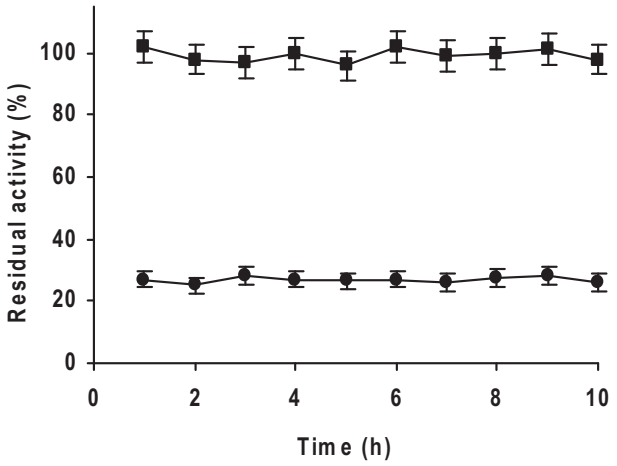


Fig. 7. Operational stability of the ficin immobilized enzyme (85 mg/g). The coagulum yield (circles) and caseinolytic activity (considering 100% the initial one) (squares) was calculated as reported in Materials and methods section.

Although some effect of the temperature (that will reduce the catalytic activity of the ficin) cannot be discarded, the main difference is that the enzymatic process is in this case performed all the time maintaining soluble the protein hydrolysate, while before, the hydrolysate precipitated very rapidly, that way it was performed in the presence of enzyme aggregates where most of the hydrolysate products were concentrated. However, still was clear the significant effect of enzyme loading more than the ficin "global concentration" (that in all experiments was identical).

3.4. Skimmed milk clotting

Finally, free ficin and the new biocatalysts have been used to produce a real milk clotting via the two steps process described above. In this case, the only data we can give is the final yield of the coagulum; Table 2 shows the values obtained. We need to use much more amount of immobilized ficin than or free enzyme in this experiment.

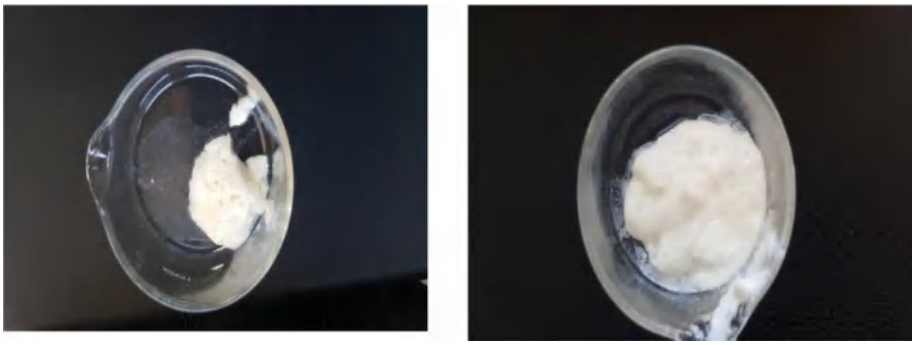
Around 20% of wet coagulum yield was obtained using free ficin. Using immobilized enzyme, the yields increased when the loading in the support increased (Table 2). Using the 85 mg ficin/g biocatalysts, a 27% coagulum yield could be achieved. The SDS-PAGE analysis of the coagulum showed that the size of the peptides were very different using free or immobilized enzyme (Fig. 5). For the free enzyme the size of the peptides was much smaller. However, it should be considered that the ficin was incorporated to the coagulum when it was used as free enzyme and not when it used in the immobilized form. That does not make possible to fully stop the reaction using the free enzyme. Among the different loaded biocatalyst, the least loaded biocatalysts gave a higher amount of the larger hydrolysate fragments.

Fig. 6 shows that the hydrolysis enzymatic step is very important for the properties of the product, as the aspect of the coagulum obtained with free or immobilized ficin is very different. The free ficin produced a weak and aqueous aggregate, while the immobilized ficin gave a denser and more solid aggregate.

That way, even requiring a higher amount of enzyme and making necessary to use a two-step clotting strategy, the use of the immobilized ficin may have some advantages to produce cheeses, mainly considering that the biocatalysts was reused for 10 cycles without detecting any change in its performance in the coagulation of milk (Fig. 7). This is expected because the very mild conditions of the reaction and the use of skinned milk, that prevent the closing of the support pores by the milk fat. The biocatalyst was not submitted to any washing step.

4. Conclusion

The results presented in this paper shows how immobilized ficin may have a great interest to produce a dense and high yield coagulum in milk clotting using skimmed milk. This requires separating the two steps of the milk clotting. The enzymatic hydrolysis of casein must be performed at 4 °C to prevent hydrolysate aggregation that can make complex the separation of the aggregate and the biocatalysts. In a second step, the hydrolysate solution is heated to 40 °C to force the hydrolysate aggregation. Clotting yields as high as 27% can be obtained using this strategy.



Coagulum obtained with immobilized ficin

Coagulum obtained with free ficin

Fig. 6. The coagula obtained by clotting of skimmed milk by immobilized (using the 85 mg/g biocatalyst) and free ficin following the conditions listed in legend of Fig. 5.

Moreover, it has been also shown that in some processes, as complex as the coagulant activity of immobilized enzymes, the use of identical amounts of immobilized enzyme but using different loadings did not give the same "activity", the relevant factor is the enzyme loading in the biocatalysts.

Acknowledgments

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Conclusion

The results presented in this paper shows how immobilized ficin may have a great interest to produce a dense and high yield coagulum in milk clotting using skimmed milk. This requires separating the two steps of the milk clotting. The enzymatic hydrolysis of casein must be performed at 4 °C to prevent hydrolysate aggregation that can make complex the separation of the aggregate and the biocatalysts. In a second step, the hydrolysate solution is heated to 40 °C to force the hydrolysate aggregation. Clotting yields as high as 27% can be obtained using this strategy.

Moreover, it has been also shown that in some processes, as complex as the coagulant activity of immobilized enzymes, the use of identical amounts of immobilized enzyme but using different loadings did not give the same “activity”, the relevant factor is the enzyme loading in the biocatalysts.

Conclusion

Les résultats présentés dans cet article montrent à quel point la ficine immobilisée peut avoir un grand intérêt pour produire un coagulum avec un bon rendement de coagulation en utilisant du lait écrémé. Cela nécessite de séparer les deux étapes de la coagulation du lait. L'hydrolyse enzymatique de la caséine doit être réalisée à 4 °C pour éviter l'agrégation de l'hydrolysat qui peut entraver la séparation de l'agrégat et des biocatalyseurs. Dans une seconde étape, la solution d'hydrolysat est chauffée à 40 ° C pour forcer l'agrégation de l'hydrolysat. Des rendements de coagulation aussi élevés que 27% peuvent être obtenus en utilisant cette stratégie.

De plus, il a également été montré que dans certains procédés, aussi complexes que l'activité coagulante d'enzymes immobilisées, l'utilisation de quantités identiques d'enzyme immobilisée mais en utilisant des charges de support différentes ne donnait pas la même «activité», le facteur pertinent est la charge enzymatique dans les biocatalyseurs.

CONCLUSION AND RECOMMENDATIONS

The objectives of this work were the preparation of an immobilised biocatalyst of ficin with improved characteristics (activity, thermal and chemical stability and stabilization in presence of chemical agents) compared to the free enzyme and for an eventual reuse of this biocatalysts in the hydrolysis of proteins (in our case we used a bovine casein).

Firstly, we proceeded with the immobilization on supports of agarose activated with glycidol (glyoxyl agarose), Divinyl sulfone (DVS-agarose) ethylenediamine (MANAE-agarose) of glutaraldehyde (glutaraldehyde-agarose). Secondly, was the chemical modification of immobilised ficin on glyoxyl agarose using glutaraldehyde and ethylenediamine (EDA) and amination of free ficin (using EDA), then its immobilization on glyoxyl agarose. Finally, we used the immobilized ficin on glyoxyl agarose to coagulate milk (as milk clotting agent).

Glyoxyl supports have some special characteristics that determine its potential, even when it only involves primary amino groups in the immobilization (Mateo *et al.*, 2007). Immobilization of enzyme on glyxyl agarose is very depended on the reactive groups on the surface of the protein (Abian *et al.*, 2004) and in the support (Mateo *et al.*, 2005). In the case of ficin, immobilization was fast; the full immobilization ($\geq$ 95% of initial activity) was obtained after 1 h (at room temperature and 50 mM carbonate buffer pH 10). However, after 3 h of immobilization, activity of ficin decreased until 60% and then to 30% after 24h. These results show that there are not areas with a high density of lysine groups in the surface of enzyme. The best preparation of this biocatalyst was after 3 h of immobilization with a good orientation to recognize large substrates (casein), with more than 60% of the initial activity using a small synthetic substrate (BANA) and large naturel substrates (casein). This immobilized biocatalyst of ficin had a significant stabilization (40-folds) compared to the free enzyme. More than 3 h Enzyme/support reaction produced a decrease in enzyme stability. This decrease in stability was caused by conformational change which caused changes in certain essential groups for enzymatic activity (e.g., the catalytic Cys). This stabilization permitted that the immobilized biocatalyst can be used under more drastic conditions in hydrolysis of casein as a higher temperature or presence of concentred urea. The operational stability of the immobilized enzyme at 55 °C and pH 7 seems very adequate for the application of this biocatalyst in hydrolysis of proteins. The

optimal loading of enzyme can be different for each substrates or application, because a high enzyme loading can induce a steric hindrance for large substrates,

Immobilization of ficin on aminated supports at pH 5,7 and 9 was very slow with a real lose in the activity. The best yield near to 10 % of initial activity was obtained at pH 9. This result shows that ficin cannot be immobilized on aminated supports, probably due to the distribution of anionic and cationic residues in the surface of ficin affecting the adsorption on the supports surface.

Using glutaraldehyde-agarose which is an heterofunctional activated support, having both ion exchange capacity and chemical reactivity, is due to the activation of primary amino groups in the support by reaction with glutaraldehyde. This support can react mainly with nonionized primary amino groups. The best result in term of immobilization yield and expressed activity was obtained at pH 7 with total immobilization after 4 h and 40% of residual activity after 24h using BANA and casein as substrates. However, at pH 5 only 20% of activity was obtained after 24h this preparation was discarded. At pH 9 total immobilization was obtained after 6h, but at 24h a significant part of activity has been lost (residual activity near to16%). This result, could be due to the relatively low stability of the glutaraldehyde groups at alkaline pH value, immobilization employing these supports is usually performed at neutral pH values. Immobilization at 25 or 200 mM sodium phosphate is similar, while the aminated support is unable to immobilize more than 30–40% of the enzyme. Stabilization depended on the pH and the substrate used to determine the residual activity. The stabilization was clear at pH 7 than pH 5 and pH 9. Using a small substates (BANA) immobilized ficin was slightly more stable than when using casein. These differences in stabilisation may be due to the different interactions between the enzyme and the supports, affecting the activity versus the different substrates. Enzyme loading had not a significant effect on immobilized enzyme stability.

Chemical modification of glyoxyl-ficine can be an interesting tool to improve the characteristics of enzymes. Modification of immobilized fine was carried out by amination using EDA or by glutaraldehyde.

Hydroxylamine incubation had very negative effect on enzyme activity (almost entire activity was destroyed) using either a modified or an unmodified preparation of ficin with both substrates (BANA and casein). Amination also had a negative effect on the enzyme activity, this effect was lower using casein as substrate comparing to using the BANA. The stability of ficin was very depended on the pH of amination. At pH 5 amination produced a slight increase on the stability. However, at pH 7 and 9 amination had a negative effect on the stability (the effect was clearer at pH 9 then at pH 7). These results show that it is possible to chemically aminate the ficin, without altering these characteristics, as a preliminary step to improve the formation of more intense multipoint covalent attachment during enzyme immobilization in order to improve the stability of the obtained biocatalyst, or even to modify the orientation of the enzyme to allow the use of fully charged supports in the hydrolysis of protein.

Glutaraldehyde modification of the immobilised ficin on glyoxyl agarose had a positive effect on enzyme activity using casein (activity increase to the double), while the effect was very negative using BANA as substrate (45% of activity was lost using 1% glutaraldehyde). Stability was significantly improved at pH 5 and 7. This stabilization may be due to the rigidification of the enzyme structure caused by the formation of the intermolecular crosslinking.

In the other hand, free ficin had been aminated using ethylenediamine and carbodiimide to transform all exposed carboxylic groups into amino groups in order to improve immobilization and stability. This treatment affect slightly activity versus both substrates (80% and 90% of residual activity using BANA and casein respectively). This decrease is caused by the autolysis of ficin during amination and dialysis. After immobilization on several conditions of the aminated ficin, the new obtained biocatalyst was compared to the biocatalyst obtained with the no-modified (no aminated) ficin. The biocatalyst of aminated ficin had improved properties compared to the one obtained with the natural ficin. Immobilization pH affect the activity and stability of the biocatalyst. For the aminated ficin the optimal biocatalyst was obtained after 1H of immobilization with the best stability and activity. After 1H immobilization, activity and stability of the aminated biocatalyst decrease, this was probably caused by some modifications in the enzyme properties. Using this biocatalyst in the hydrolysis of casein, result showed that activity was improved comparing with the natural biocatalyst, in the other hand stability was clearly improved compared to the natural one permitting its use in unsuitable conditions

for enzymatic activity as higher temperature and higher concentration of Urea (8M). Concerning the loading of the support amination con not affect the maximum loading without causing a steric hindrance and a loss of activity versus natural large substrates (casein).

Ficin immobilized and stabilized via multipoint covalent attachment on glyoxyl agarose beads may be considered useful for some applications. As showed, ficin immobilized over glyoxyl agarose had a higher proteolytic or caseinolytic activity. Cheese making with non-specific proteases as ficin can present some organoleptic problems on the final product as bitterness and undesirable texture caused by the intense hydrolysis of milk protein. This problem can be solved using immobilised proteases. The result showed that immobilised ficin has a great interest in the milk clotting applications. Using skimmed milk, and two step casein coagulation an enzymatic step caried out at 4ºC to prevent the formation of coagulum by aggregation of destabilized casein (causing difficulties in the separation and recovery of biocatalysts) and second step of coagulation at 40ºC. Clotting yields was significatively better using immobilized enzyme (the best yield was around 27%) compared to the yield using a free ficin (approximatively 20%). Using different loadings of the biocatalyst did not give the same activity for the same amounts of immobilized enzyme. The more loaded biocatalyst was the most active and gave the best yield (yield was 19 % with the support loaded at 10 mg ficin per gram of support, 24% and 27 % respectively for the support loaded with 30 mg/g and 100 mg/g enzyme/supports). Moreover, it has been also shown that with less loaded supports milk clotting activity was almost undetectable. The study of the hydrolysis with SDS PAGE showed that hydrolysis of casein was less while using immobilized ficin than using free enzyme. These results make these new ficin biocatalysts very promising ones in the cheese making industries.

Further studies should be carried out using the ficin biocatalyst.

- Analysing the enzyme load in the enzyme activity for each likely application of the immobilized ficin,
- Use of this biocatalyst for the hydrolysis of other substrates with several undesirable or critique condition,
- Co-immobilization of ficin with other enzymes (proteases or other enzyme) and its use in industrial application,
- Use of ficin biocatalyst in the production of sane specific cheeses,

- Use of these biocatalysts in the biotransformation and controlled synthesis (amin acid, bioactive peptides, antibody).
- Use of these biocatalysts in the pharmaceutical application.

Les principales conclusions tirées de se travail qui avait pour objectifs la préparation d'un biocatalyseur de ficine immobilisé avec des caractéristiques améliorées (activité, stabilité thermique et chimique et stabilisation en présence d'agents chimiques) par rapport à l'enzyme libre ainsi qu'une éventuelle réutilisation de ces biocatalyseurs dans l'hydrolyse des protéines (dans notre cas nous avons utilisé une caséine bovine). Premièrement, nous avons procèdea l'immobilisation sur de l'agarose activé avec le glycedol (glyoxyl agarose), éthylènediamine (MANAE- agarose) du glutaraldéhyde (glutaraldéhyde-agarose). Deuxièmement, les modifications chimiques de la ficine immobilisée sur glyoxyl agarose à l'aide de glutaraldéhyde et éthylènediamine (EDA) et amination de la ficine libre (à l'aide d'EDA), puis son immobilisation sur le glyoxyl agarose. Enfin, nous avons utilisé la ficine immobilisée sur glyoxyl agarose pour coaguler le lait (comme agent de coagulation du lait).

Le support actives avec le glycedol a des caractéristiques particulières qui déterminent son potentiel, même lorsqu'elle n'implique que des groupes amino primaires dans l'immobilisation (Mateo et al., 2007). L'immobilisation de l'enzyme sur le glyxyl-agarose dépend fortement des groupes réactifs à la surface de la protéine (Abian et al., 2004) et dans le support (Mateo et al., 2005). Dans le cas de la ficine, l'immobilisation a été rapide ; l'immobilisation complète (≥ 95% d'activité initiale) a été obtenue après 1 h (à température ambiante et tampon carbonate 50 mM pH 10). Cependant, après 3 h d'immobilisation, l'activité de la ficine a diminué jusqu'à 60% et puis à 30% après 24h. Ces résultats montrent qu'il n'y a pas de zones à forte densité de groupes de lysine à la surface de l'enzyme. La meilleure préparation de ce biocatalyseur est obtenue après 3 h d'immobilisation avec une bonne orientation de site actif pour reconnaître les gros substrats naturelle (caséine), avec une activité résiduelle de plus de 60% en utilisant un petit substrat synthétique (BANA) et les grands substrats naturels (caséine). Ce biocatalyseur de la ficine avait une stabilisation significative (40 fois) par rapport à l'enzyme libre. Plus de 3 h de contacte ou réaction enzyme / support provoque une diminution de la stabilité de l'enzyme. Cette diminution de la stabilité a été causée par un changement de conformation qui provoque des changements dans le site catalytique. La stabilisation obtenues avec cette immobilisation permis l'utilisation de cette enzyme immobilisée dans des condition d'hydrolyse des caséine très extrêmes telle que la température élevée et la présence d'agent chimique telle que l'urea. Le biocatalyseur de ficine obtenu est très adéquat pour une application dans l'hydrolyse des proteines a pH 7 et a 55º C

La chargeme optimal de support en l'enzyme peut être différente pour chaque substrat ou application, car une charge enzymatique élevée peut induire un encombrement stérique pour les grands substrats tel les caséines.

L'immobilisation de la ficine sur des supports aminés à pH 5,7 et 9 a été très lente avec une grande perte en activité. Le meilleur rendement obtenu avec ce support était proche de 10% d'activité résiduelle obtenu à pH 9. Ce résultat montre que la ficine ne peut pas être immobilisée sur des supports aminé, probablement en raison de la distribution de résidus anioniques et cationiques dans la surface de la ficine affectant l'adsorption sur la surface des supports.

En utilisant le support activé avec du glutaraldéhyde qui donne un support hétérofonctionnel, ayant à la fois une capacité d'échange d'ions et une réactivité chimique élevée, due à l'activation de groupement amine primaires dans le support par réaction avec le glutaraldéhyde. Ce support peut réagir principalement avec les groupements amino primaires nonionisés. Le meilleur résultat en termes du rendement d'immobilisation et l'activité résiduelle ont été obtenus à pH 7 avec une immobilisation totale après 4h de contacts ; l'activité résiduelle était de 40% après 24 h d'immobilisation en utilisant le BANA et la caséine comme substrats. Cependant, à pH 5, seulement 20% d'activité ont été obtenus après 24 h cette préparation a été rejetée. A pH 9, une immobilisation totale a été obtenue après 6 h, mais à 24h mais une perte significative de l'activité a été observée (activité résiduelle proche de 16%). Ce résultat pourrait être dû à la stabilité relativement faible des groupes glutaraldéhyde à pH alcalin, l'immobilisation en utilisant ces supports est généralement effectuée à valeurs de pH neutres. L'immobilisation dans le tampon phosphate a 25 ou 200 mM est similaire, tandis que le support aminé est incapable d'immobiliser plus de 30 à 40% de l'enzyme. La stabilisation dépendait du pH et du substrat utilisé pour déterminer l'activité résiduelle. La stabilisation était très claire à pH 7 que pH 5 et pH 9. En utilisant le substrat synthétique (BANA)la stabilité était légèrement plus élevée que lors de l'utilisation de la caséine. Ces différences de stabilisation peuvent être dues aux différentes interactions entre l'enzyme et les supports, affectant l'activité par rapport aux différents substrats. La charge enzymatique n'avait pas d'effet significatif sur la stabilité de l'enzyme immobilisée.

La modification chimique de la ficine immobilisée sur le glyoxyl-agarose peut être un outil intéressant pour améliorer les caractéristiques des enzymes. La modification de la ficine immobilisée a été effectuée par amination en utilisant le EDA ou par glutaraldéhyde.

L'incubation dans hydroxylamine a eu un effet très négatif sur l'activité enzymatique (presque toute l'activité a été perdue) en utilisant soit une préparation modifiée ou non

modifiée de ficine avec les deux substrats (BANA et caséine). L'amination a également produit un effet négatif sur l'activité enzymatique, cet effet était plus faible en utilisant la caséine comme substrat par rapport à l'utilisation du BANA. La stabilité de la ficine dépendait fortement du pH de l'amination. À pH 5 l'amination provoque une légère augmentation de la stabilité. Cependant, à pH 7 et 9, l'amination avait un effet négatif sur la stabilité (l'effet était plus clair à pH 9 puis à pH 7). Ces résultats montrent qu'il est possible de modifier chimiquement la ficine par amination, sans altérer ces caractéristiques comme étape préliminaire pour améliorer la formation de liaisons multi-covalentes ce qui intensifier la fixation lors de l'immobilisation permettant d'améliorer la stabilité du biocatalyseur obtenu, voire de modifier l'orientation de l'enzyme pour permettre l'utilisation de supports chargés dans l'hydrolyse des protéines.

La modification de la ficine immobilisée sur le glyoxyl-agarose avec le glutaraldéhyde avait un effet positif sur l'activité enzymatique en utilisant la caséine (augmentation de l'activité au double), tandis que le l'effet était très négatif en utilisant BANA comme substrat (45% de l'activité a été perdue en utilisant 1% glutaraldéhyde). La stabilité a été considérablement améliorée à pH 5 et 7. Cette stabilisation peut être due à la rigidification de la structure enzymatique causée par la formation des réticulations intermoléculaire.

D'autre part, la ficine libre avait été aminée à l'aide d'éthylènediamine et de carbodiimide pour transformer tous les groupes carboxyliques exposés en groupes amino afin d'améliorer l'immobilisation et la stabilité. Ce traitement affecte légèrement l'activité par rapport aux deux substrats (80% et 90% de l'activité résiduelle en utilisant respectivement BANA et caséine). Cette diminution est causée par l'autolyse de la ficine pendant l'amination et la dialyse. Après immobilisation de la ficine aminée dans plusieurs conditions, le nouveau biocatalyseur obtenu a été compare avec les biocatalyseurs obtenue evec le biocatalyseur obtenu avec la ficine non aminee. Le biocatalyseur obtenu avec la ficine aminée avait des propriétés améliorées par rapport à celui obtenu avec le ficine naturelle. Le pH d'immobilisation affecte l'activité et la stabilité du biocatalyseur. Pour la ficine aminée le biocatalyseur optimal a été obtenu après 1H d'immobilisation avec une meilleure stabilité et activité. Après immobilisation pendent 1H, l`activité et stabilité de biocatalyseur aminé diminue, probablement causée par certaines modifications des propriétés de l'enzyme. En utilisant ce biocatalyseur dans l'hydrolyse de la caséine, le résultat a montré que l'activité a été améliorée par rapport au biocatalyseur naturel, la stabilité était aussi nettement améliorée par rapport au biocatalyseur naturel permettant son utilisation dans des conditions inadaptées pour l'activité enzymatique telle que les températures élevées et des concentration

élevée d'urée (8M). L'amination n'affecte pas la charge maximale de support en enzyme sans provoquer un encombrement stérique et une perte d'activité par rapport aux grands substrats naturels (caséine).

La ficine immobilisée et stabilisée par des liaisons multi-covalente sur glyoxyl-agarose peut être considérées utiles pour certaines applications. Comme montré, la ficine immobilisée sur le glyoxyl agarose avait une activité protéolytique ou caséinolytique plus élevée. La fabrication des fromages avec des protéases non spécifiques comme la ficine peut présenter des problèmes organoleptiques sur le produit final (amertume et de texture indésirable) provoquée par l'hydrolyse intense des protéines de lait. Ce problème peut être résolu en utilisant des protéases immobilisées. Le résultat a montré que la ficine immobilisée présente un grand intérêt dans les applications de coagulation du lait. Utilisant lait écrémé et coagulation en deux (étapes une étape enzymatique réalisée à 4 ° C pour empêcher la formation de coagulum par agrégation de caséine déstabilisée (causant des difficultés dans la séparation et la récupération des biocatalyseurs) et deuxième étape de la coagulation à 40 ° C. Les rendements de coagulation étaient significativement meilleurs en utilisant l'enzyme immobilisée (le meilleur rendement était d'environ 27%) par rapport au rendement avec une ficine libre (environ 20%). L'utilisation de déférente charge de biocatalyseur n'a pas donné la même activité pour les mêmes quantités d'enzyme immobilisée. Le biocatalyseur le plus chargé était le plus actif et donnait le meilleur rendement (le rendement était de 19% avec le support chargé à 10 mg de ficine par gramme de support, 24% et 27% respectivement pour le support chargé à 30 mg / g et 100 mg / g enzyme / supports). De plus, il a également été démontré qu'avec des supports moins chargés, l'activité de coagulation était presque indétectable. L'étude de l'hydrolyse avec SDS PAGE a montré que l'hydrolyse de la caséine était faible avec la ficine immobilisée qu'avec l'utilisation libre. Ces résultats rendent ces nouveaux biocatalyseurs de ficine très prometteurs dans les industries de fabrication du fromage.

D'autres études devraient être menées pour le biocatalyseur de ficine.

- analyse de l'effet de la charge enzymatique sur l'activité pour chaque application probable de la ficine immobilisée,

- Utilisation de ce biocatalyseur pour l'hydrolyse d'autres substrats sous des conditions indésirables ou critiques,

- Co-immobilisation de la ficine avec d'autres enzymes (protéases ou autre enzyme) et son utilisation en application industrielle,

- Utilisation du biocatalyseur de ficine dans la production de fromages spécifiques,

- Utilisation de ces biocatalyseurs dans la biotransformation et la synthèse contrôlée (acide aminé, peptides bioactifs, anticorps).

- Utilisation de ces biocatalyseurs dans l'application pharmaceutique.

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Abstract

Immobilization and chemical modification of the free or immobilized enzyme are among the most proposed solutions to overcome same enzyme limitations and improve the performance. In this work, Ficin extract was immobilized on glyoxyl, aminated and glutaraldehyde-activated agarose. In the other hand, we studied the effects of chemical modification using glutaraldehyde, carbodiimide and ethylenediamine on the properties of immobilized/stabilized ficin on glyoxyl agarose and free ficin. Finally, we use the immobilized ficin for the milk coagulation.

Immobilization of ficin on glyoxyl agarose was performed at pH 10 and 25°C for 3 hours maintaining 60% of activity using BANA (Benzoyl-arginine-*p*-nitroanilide) and casein at 55 ºC. The obtained biocatalyst was active and stable over a large range of pH and temperatures. The obtained biocatalyst was three times more active compared to the free enzyme in presence of 2 M at 55°C and 65°C and could be reused for fives cycles at 55 ºC without losing activity in hydrolysis of casein.

Using aminated supports immobilization yield was very low using both substrates and no effect in enzyme stability. Using aminated supports activated with glutaraldehyde, immobilization was optimal at pH 7 with full immobilisation and 40% of maintained activity using BANA and 30% using casein. Stabilization of ficin was observed after immobilization. The maximum loading obtained was 70 mg/g of supports and more than 30 mg/g activity versus casein decrease using casein and not affected using BANA (due to the steric hindrances).

The effect of amination using carbodiimide and ethylenediamine was reported at pH 9, where activity increased over 10% using casein and decreased more than 5 folds using BANA. The specificity of the enzyme has been strongly affected (stability was increased at pH 5 and decreased at pH9). On the other hand, free aminated ficin, retained around 80% of activity versus BANA and 90% versus casein. After optimization a new biocatalyst was compared to the obtained using the non-aminated enzyme. The activity decreased versus BANA, but it increased versus casein. Stability of the new biocatalyst was increased more than the non-aminated ficin biocatalyst mainly at pH 7. This permitted to have more hydrolysis of casein at pH 7 and 66 ºC compared to the unmodified enzyme. Glutaraldehyde treatment decreased the activity versus BANA while it improved the activity versus casein. This treatment improves also the thermostability of ficin at pH 7.

Besides that, ficin immobilized over glyoxyl agarose with different loading was used in milk coagulation, the remaining activity versus casein was 60%, 58%, 41% and 14%, respectively for 3, 10, 30 and 85mg ficin per gram supports due to casein diffusional limitations. Using the biocatalyst in the two-step milk clotting (enzymatic step at 4ºC to prevent aggregation of hydrolysed casein and then at 40ºC), the yield was 9%, 24%, 27% and 20% for the 10mg/g, 30 mg/g and 85 mg/g immobilized ficin and free ficin respectively, under similar ficin concentrations. These results make these ficin biocatalysts very promising for several application.

Keywords: ficin, agarose, immobilization, stabilization, performance, chemical modification, milk clotting.

Résumé

L'immobilisation et les modifications chimiques des enzyme immobilisée ou non immobilisée sont parmi les solutions les plus approprier pour améliorer leurs performances. Dans ce travail, la ficine a été immobilisée sur de l'agarose modifié (glyoxyle, aminé et glutaraldéhyde). D'autre part, nous avons étudié les effets des modifications chimique en utilisant le glutaraldéhyde, le carbodiimide et l'éthylènediamine sur les propriétés de la ficine immobilisée sur le glyoxyle agarose et sur la ficine libre. Enfin, nous avons utilisé la ficine immobilisée dans la coagulation du lait.

L'immobilisation de la ficine sur le glyoxyl agarose a été réalisée à pH 10 et 25°C pendant 3 heures. L'activité résiduelle était de 60% en utilisant le BANA (Benzoyl-arginine-p-nitroanilide) et la caséine à 55 ºC. Le biocatalyseur obtenu était actif et stable sur une large gamme de pH et de températures avec une activité trois fois superieure que l'enzyme libre en présence 2 M de l'urée à 55°C et 65°C et pouvait être réutilisé dans cinq cycles d'hydrolyse des caséines à 55 ºC sans perdre son activité.

L'immobilisation sur des supports aminés a était très faible et n'a eu aucun effet sur la stabilité de l'enzyme. En utilisant des supports aminés activés avec du glutaraldéhyde, l'optimum est obtenu à pH 7 avec une activité résiduelle de 40% et 30% en utilisant le BANA et les caséines respectivement avec une amélioration de la stabilité. Plus de 30 mg de ficine/g de support l'activité de l'enzyme diminue avec les caséines et n'était pas affecter avec le BANA (en raison des encombrements stériques).

L'amination avec carbodiimide et l'éthylènediamine à pH 9 a augmenté de plus de 10% l'activité avec la caséine et a diminué de plus de 5 fois avec le BANA. La spécificité de l'enzyme a été fortement affectée (la stabilité a augmenté à pH 5 et diminué à pH 9). La ficine libre aminée a conservé une activité de 80 % avec le BANA et 90% avec les caséines. Après optimisation, l'activité de nouveau biocatalyseur a diminuée avec le BANA et améliorée avec les caséines en comparaison avec l'enzyme non aminée. La stabilité a été améliorée principalement à pH 7, ce qui a permis d'avoir une hydrolyse plus importante de la caséine à pH 7 et 66 °C par rapport à l'enzyme non modifiée. Le traitement au glutaraldéhyde a amélioré la thermostabilité à pH 7 et l'activité avec les caséines mais diminue l'activité contre le BANA.

La ficine immobilisée sur de glyoxyl agarose avec différentes charges a été utilisée pour la coagulation du lait, l'activité résiduelle était de 60%, 58%, 41% et 14%, respectivement pour 3, 10, 30 et 85 mg de ficine/g de support en raison des limitations de diffusion de la caséine. La coagulation du lait en deux étapes (étape enzymatique à 4ºC pour empêcher l'agrégation de la caséine hydrolysée, puis à 40ºC) a donnée un rendement de 9 %, 24 %, 27 % et 20 % pour les 10 mg/g, 30 mg/g et 85 mg/g et la ficine libre respectivement pour la même concentration en ficine. Ces résultats rendent ces biocatalyseurs de ficine très prometteurs pour plusieurs applications.

Mots clés : ficine, agarose, immobilisation, stabilisation, performances, modification chimique, amination, coagulation du lait.

خلاصة

يعد تثبيت الإنزيمات والتعديل الكيميائي عليها سواء كانت حرة أم ثابتة من بين الحلول الأكثر ملائمة لتحسين قدراتها. في هذا العمل، تم تثبيت الفيسين على أمين جليوكسيل الأغاروز المنشط باالغلوتارالدهيد. ومن جهة أخرى، قمنا بدراسة آثار التعديل الكيميائي باستخدام الجلوتارالدهيد، الكربوديميد والإيثيلين دياامين على خواص الفيسين المثبت على جليوكسيل الاغاروز وعلى الفيسين الحر. وفي النهاية تم إستخدام الفيسين المثبت كمخثر للحليب. تثبيت الفيسين على جليوكسيل الاغاروز تم في حموضة pH 10 وفي حرارة 25 درجة مئوية لمدة 3 ساعات. تم الحفاظ على 60 ٪ من النشاط الإنزيمي باستخدام BANA (بنزويل-أرجينين- نيتروانيليد) والكازين في حرارة 55 درجة مئوية. المحفز الحيوي المحصل عليه وجد نشطًا ومستقرًا في نطاق واسع من الحموضة و الحرارة. كما وجد المحفز الحيوي أكثر نشاطًا بثلاث مرات مقارنة بالأنزيم الحر في وجود 2 مولار من اليوريا في حرارة 55 و 65 درجة مئوية مع إمكانية إعادة استخدامه لخمس دورات في حرارة 55 درجة مئوية دون اي فقدان لنشاط تحليل الكازين. مردود التثبيت على الدعامات الأمينية كان ضعيفا للغاية مع كلا الركيزتين كما لم يكن له اي تأثير على إستقرار الإنزيم. التثبيت باستخدام الدعامات الأمينية المنشطة بالجلوتارالدهيد كان مثاليا في حموضة pH 7 حيث تم تثبيت كلي للإنزيم مع الحفاظ على 40٪ من النشاط باستخدام BANA و 30٪ من النشاط باستخدام الكازين, كما لوحظ استقرار للفيسين بعد التثبيت. الحد الأقصى للتعبئة هو 70 مجم / جرام من الدعامات. مع تعبئة 30 مجم / جرام تم تسجيل انخفاض في النشاط مع الكازين دون ان يتأثر باستخدام BANA (بسبب العوائق الستريكية). تمت دراسة تأثير الأمننة (amination) باستخدام الكربوديميد و والإيثيلين دياامين في حموضة pH 9، حيث زاد النشاط بأكثر من 10 ٪ باستخدام الكازين وانخفض أكثر من 5 أضعاف باستخدام BANA. تأثرت خصوصية الإنزيم بشدة (زاد الاستقرار عند الحموضة pH 5 وانخفض عند الحموضة pH 9). من ناحية أخرى ، احتفظ الفيسين الأميني الحر بحوالي 80٪ من النشاط مع BANA و 90٪ مع الكازين. بعد التحسين، تمت مقارنة محفز حيوي جديد مع المحفز المحصل عليه باستخدام الإنزيم غير الأميني. تم تسجيل انخفاض للنشاط مع BANA ، ولكنه ازداد مع الكازين. استقرار المحفز الحيوي الجديد كان أكثر من استقرار المحفز الحيوي غير الأميني خاصة في حموضة pH 7. مما سمح بالحصول على مزيد من نشاط تحليل الكازين في حموضة pH 7 وحرارة 66 درجة مئوية مقارنة بالإنزيم غير المعدل. من جهة اخرى العلاج بالجلوتارالدهيد قلل النشاط مع BANA بينما أدى إلى تحسينه مع الكازين. عمل هذا العلاج أيضًا على تحسين استقرارالفيسين في الحرارة في حموضة pH 7. بالإضافة إلى ذلك ، تم استخدام الفيسين المثبت فوق غليوكسيل الاغاروز في عملية تخثير الحليب، وكان النشاط المتبقي مع الكازين 60٪ و 58٪ و 41٪ و 14٪ على التوالي لدعامات 3 و 10 و 30 و 85 ملجم فيسين لكل جرام بسبب ضعف انتشار الكازين. استخدام المحفز الحيوي في عملية تخثير الحليب المكونة من خطوتين (خطوة إنزيمية عند 4 درجات مئوية لمنع تراكم الكازين المتحلل بالماء ثم عند 40 درجة مئوية) ، كان الناتج 9٪ ، 24٪ ، 27٪ و 20٪ لـ 10 مجم / جم ، 30 مجم / جم و 85 مجم / جم بالنسبة للفيسن المثبت و الحر على التوالي، تحت تركيزات مماثلة من الفيسين. هذه النتائج تجعل المحفزات الحيوية للفيسين واعدة للغاية مع إمكانية استخدامها في العديد من التطبيقات.

الكلمات المفتاحية: فيسين، أغاروز، تثبيت، استقرار،قدرة ، تعديل كيميائي ، تخثر الحليب