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Ficin: a protease extract with relevance in biotechnology and biocatalysis

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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.

Key-words: Proteases, proteolysis, synthesis of peptides, bioactive peptides, protease immobilization, ficin

The layout of the current review is:

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2.- Ficin immobilization

3.- Ficin applications

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3.8.1.- Use of ficin as anti-parasite agent.

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

5.- Conclusion and future trends

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 an 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 are 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] (Figure 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] (Figure 42).

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 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 nine 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 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 (Figure 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 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] (Figure 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 ºC 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 ficin 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 ficin on synthetic fibers 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 ficin

3.8.1.- Use of ficin 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 ficin 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 ficin

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.

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Figure Legends

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

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

Figure 3. Different applications of ficin.

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

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.