catalysts

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Review

Genipin as An Emergent Tool in the Design of Biocatalysts: Mechanism of Reaction and Applications

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Abstract: Genipin is a reagent isolated from the *Gardenia jasminoides* fruit extract, and whose low toxicity and good crosslinking properties have converted it into a reactive whose popularity is increasing by the day. These properties have made it widely used in many medical applications, mainly in the production of chitosan materials (crosslinked by this reactive), biological scaffolds for tissue engineering, and nanoparticles of chitosan and nanogels of proteins for controlled drug delivery, the genipin crosslinking being a key point to strengthen the stability of these materials. This review is focused on the mechanism of reaction of this reagent and its use in the design of biocatalysts, where genipin plays a double role, as a support activating agent and as inter- or intramolecular crosslinker. Its low toxicity makes this compound an ideal alterative to glutaraldehyde in these processes. Moreover, in some cases the features of the biocatalysts prepared using genipin surpassed those of the biocatalysts prepared using other standard crosslinkers, even disregarding toxicity. In this way, genipin is a very promising reagent in the design of biocatalysts.

Keywords: genipin; biocatalyst; inter and intramolecular crosslinking; support activation; enzyme immobilization

1. Introduction

1.1. Biocatalysis and Biocatalysts Design

The relevance of enzymatic biocatalysis is continuously growing due to the advantages of enzymes: very high activity, selectivity and specificity under environmentally friendly conditions [1–3]. Thus, they become very suitable from the point of view of green chemistry and atomic economy [4–7]. However, enzymes have these very good properties in their physiological function and reaction medium, in many instances very different from the ones where they may have industrial interest [8–10]. From this perspective, in most cases, enzyme properties need to be improved before their implementation.

Nowadays, this may be reached by using very diverse strategies. Metagenomics [11–14] permit the use of any enzyme, even belonging to a non-cultivable organism or a no longer existing one, making the quest for suitable enzymes within reach since it permits the use of all past and present biodiversity. Genetic tools like site-directed mutagenesis or directed evolution [9,15–18] permit to further improve enzyme properties. These features may be further improved by physicochemical tools. Chemical modification of enzymes has been utilized to alter the physical properties of the enzyme surface and, thus, alter their properties, or to introduce inter- or intramolecular chemical crosslinkings, to increase the rigidity of the enzyme structure and its stability, or to prevent the dissociation of multimeric enzymes [19–23]. Coating of the enzyme surface with polymers prevents proteolysis and interaction with external interfaces (e.g., drops of solvents or gas bubbles) [24–26]. Physical crosslinking with ionic polymers has also proved to be effective in the stabilization of multimeric enzymes and in the prevention of interactions with external interfaces [27]. Enzyme immobilization was firstly developed to permit the reuse of enzymes, which are soluble molecules [28,29]. Currently, an immobilization protocol should be valid to improve some enzyme properties. Stabilization may be accomplished via multipoint covalent attachment [30]; multimeric enzymes may be stabilized via multisubunit immobilization that avoids subunit dissociation, in some instances the microenvironment generated around the immobilized enzyme molecule can have some positive effects on its stability (e.g., partitioning some deleterious reagents, like hydrogen peroxide, oxygen or organic solvents) [31–33]. In other cases, enzyme activity may be improved, in some instances by fixing more active enzyme conformations (e.g., the case of lipases interfacially activated versus hydrophobic supports), in others by making more resilient enzymes under the measurement conditions. These effects of immobilization on enzyme activity have been reviewed [34–41]. Immobilization may also improve enzyme selectivity or/and selectivity in the industrially-targeted process, reduce enzyme inhibition, increase enzyme purity, etc. [42]. As a result, immobilization is nowadays not a tool to reuse enzymes, and remains as a very important step in most biocatalytic processes, even if in some instances the price of the enzyme may seem low enough. Immobilization can be coupled to any of the other strategies to get a more suitable enzyme, as they can be used all together. In fact, there are some cases where genetic or chemical modifications have been designed to improve enzyme immobilization more than the features of the soluble enzyme [43].

This review will focus on the use of these physicochemical tools to improve enzyme features. In enzyme immobilization, a critical step is the selection of a suitable support and immobilization protocol. There are many immobilization protocols but only some selected few are suitable for taking full advantage of the immobilization (i.e., permitting an intense multipoint covalent attachment). Glyoxyl supports have proved to be able to give very intense multipoint covalent attachment involving primary amino groups of a protein, but they need to be used at pH 10 to exploit this advantage and a final reduction with sodium borohydride is required, making the method difficult to implement [44–46]. Epichlorohydrin and some di-epoxides may be used to activate supports with epoxy groups, this group may react with many different groups of a protein but at a low reaction rate, making them interesting but not ideal ones, although some heterofunctional supports have given good results [47–49]. Divinylsulfone has been recently recognized as a very suitable support activator, that permits very intense multipoint covalent attachment, but it is relatively toxic [50–52]. Glutaraldehyde is perhaps the most utilized reagent for enzyme immobilization, as it can react with primary amino groups located in a support and the resulting supports have low capability to react with the primary amino groups of a protein [53]. Although the exact mechanism of action of glutaraldehyde is not fully understood, this method is very versatile and permits very different immobilization protocols, in many instances with significant stabilizing effects [54–57]. Glutaraldehyde is also the most used reagent to introduce enzyme intermolecular crosslinkings [54,58,59]. However, the FDA does no longer permit its use. That way there is a great interest in using alternative support activating and crosslinking reagents.

1.2. Genipin Uses

In this context, genipin (Figure 1) has attracted the interest of researchers worldwide. This natural compound has many uses. For example, it is used as intermediate for the synthesis of some alkaloids [60,61]. It has been found in *Gardenia jasminoides* fruit extract and has good crosslinking properties [62,63], while its acute toxicity is low [64–66], Thus is the reason for that makes genipin having a growing number of uses and applications. Genipin (methyl 1-hydroxy-7-(hydroxymethyl)-1,4a,5,7a-tetrahydrocyclopenta [c]pyran-4-carboxylate, Figure 1) is a colorless iridoid (cyclopentapyrane-type monoterpenoid) obtained via the enzymatic hydrolysis of geniposide, one of the primary active principles contained in the fruit of *Gardenia jasminoides*.

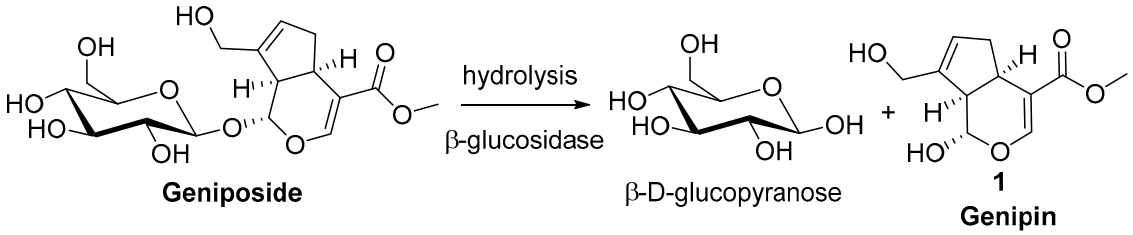

Figure 1. Hydrolysis of geniposide for the production of genipin.

2. Chemisty of Genipin

Genipin reacts with primary and secondary amines; this reaction is very useful for polymer chemistry and in many other biological applications, because its biodegradability and low toxicity make genipin an excellent reagent for cross-linking processes [67].

In spite of the high industrial applicability of genipin and the great number of processes in which it has been used, a full characterization of its structure has not been available until recently [68,69]. In fact, the structure of genipin in solid state was described by Trevor et al. (2008), assigning the absolute configuration of the three sterocentres of the molecule as 1S, 4aR, 7aR [70]. Di Tommaso et al. (2013) reported a detailed study (using 1H-NMR and corroborated by computational chemistry) of the structure of genipin in solution at different pH values [68]. These authors described that at highly alkaline pH values (around 13) genipin is not stable and undergoes self-polymerization; while in slightly alkaline medium (pH = 8) 1H-NMR signals indicate the existence of a single compound in which both rings are fused following a *cis* stereochemistry. This confirms the previously reported stereochemistry 1S, 4aR, 7aR [70] (1, Figure 2), although also the possible enantiomer ent-1 could obviously produce the same signals.

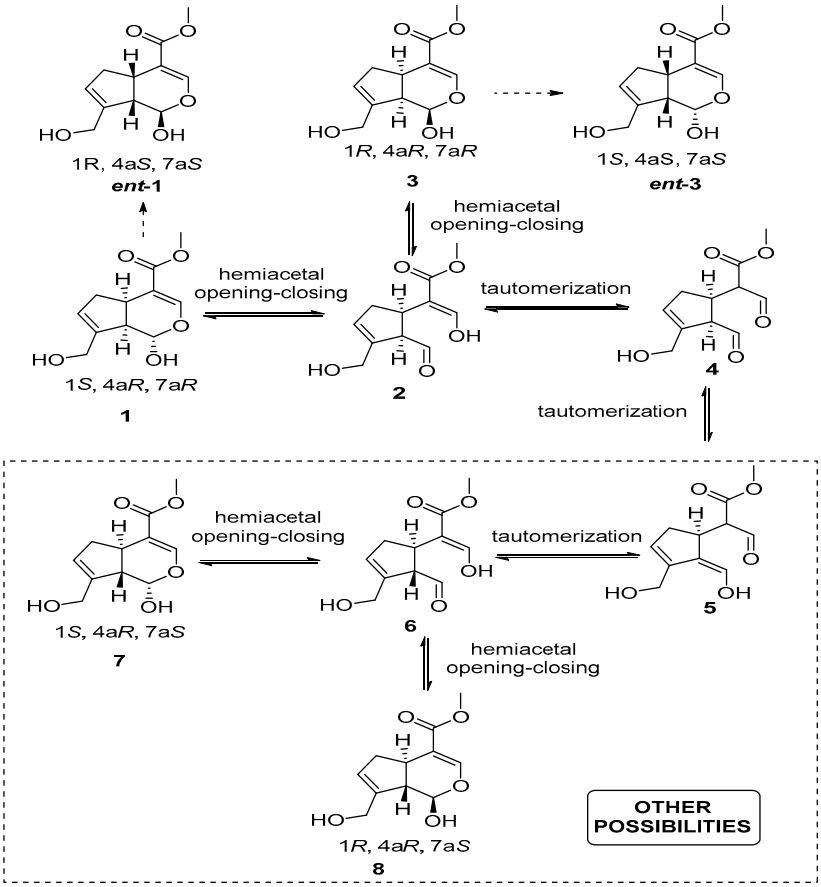

Figure 2. Different chemical structures to obtain genipin.

In acidic medium (pH = 3.6, measured in D2O/DCl), a second isomer form of genipin is detected, albeit at low concentration. This minor compound is characterized by a two *cis* coupling constants, between the two protons linked to carbons 4a and 7a, and between the protons linked to carbon 7a and 1. Therefore, this fact is pointing towards the presence of a structure such as 3, possessing the absolute configuration 1R, 4aR, 7aR, or its enantiomer ent-3. Some other possible cyclic stereoisomers such as 7 or 8 (Figure 2) were not detected. These results were ratified by molecular modeling, confirming that isomer 1 (or ent-1) are more stable than 3 (or ent-3), also establishing the most stable conformation (two half chairs) of 1 (denoted as up), in which carbon 1 is out-of-plane with respect to the plane defined by the chain bond to chiral center 4a (atoms 4, 3, 2). This up conformation is 4 kcal mol^{-1} more stable than the corresponding in-plane down conformation.

These authors also performed a study with the aim of understanding the opening mechanism of the cyclic structure of genipin 1 in solution leading to dialdehyde 4. Thus, they separately studied the energy barriers needed in the transformation of 1 into 2, and from 2 into 4, as depicted in Figure 3.

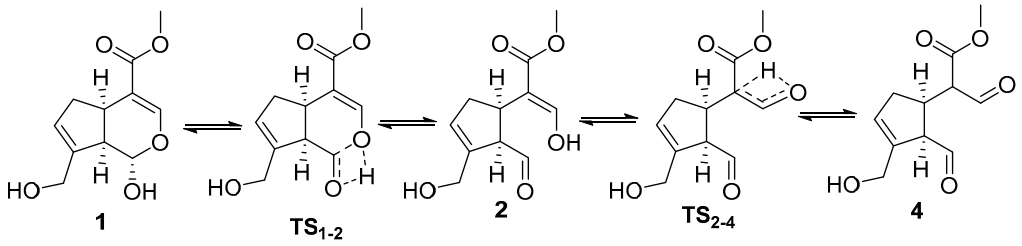

Figure 3. Mechanism for the opening of hemiacetal 1 to yield dialdehyde 4.

The first step involves an intramolecular hydrogen transfer first through a four-membered ring transition state $TS_{1\text{-}2}$ (Figure 3), leading to the intermediate 2, possessing a carbonyl and an enol moieties. This process is thermodynamically disfavored, as it is endothermic by about 15 kcal mol^{-1}, also requiring a very high activation barrier of about 48 kcal mol^{-1}. Nevertheless, if the contribution of one (six-members ring $TS_{1\text{-}2}(H_2O)$) or two (eight-members ring $TS_{1\text{-}2}(H_2O)_2$) is considered, as shown in Figure 4, the activation energy drops to about 29 and 17 kcal mol^{-1}, respectively, due to the decrease of the ring strain. In addition, the process becomes less endothermic (5.6 kcal mol^{-1}) when two molecules of water are added [68].

Similarly, the conversion of 2 into 4 via transition state $TS_{2\text{-}4}$ (Figure 3) was also modelled [68], reporting an activation enthalpy of 60 kcal mol^{-1} (no water involved) which is reduced to 22.3 kcal mol^{-1} when two water molecules are involved. On the other hand, the endothermicity of the reaction increased from −4 (no water) up to −2 kcal mol^{-1} (2 explicit water molecules). Remarkably, for the conversion of 2 into 4 the activation barrier decreased down to 16.5 kcal mol^{-1} when considering the presence of a solvent shell (ethanol was simulated by a continuum) and two water molecules. This is contrary to what was observed for $TS_{1\text{-}2}$, where the contribution of solvation (0.2 kcal mol^{-1} on the barrier and 0.6 kcal mol^{-1} for the product stability) was almost negligible.

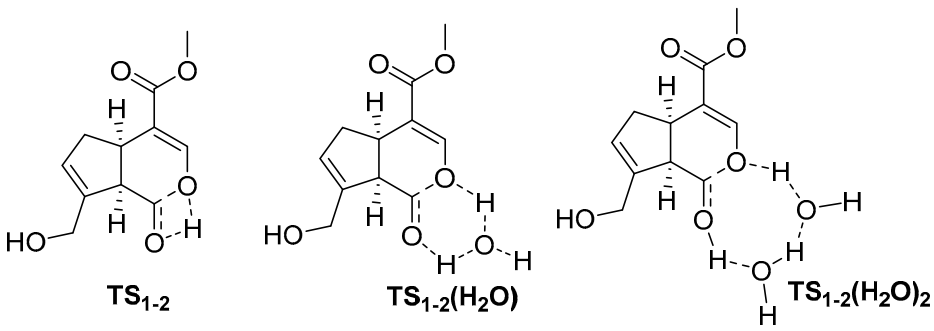

Figure 4. Different transition states in the opening of hemiacetal 1, with or without the involvement of one or two water molecules.

Reactivity of Genipin as Cross-Linker Agent

Fujikawa et al. (1998) described the formation of dimers of genipin in the presence of glycine [71]. This fact opened the door for promoting the use of genipin for covalently crosslinking of proteins containing residues with primary amine groups [67,72]. The mechanism for this crosslinking is not clear yet, and different hypotheses have been proposed; thus, Butler et al. (2003) by studying the reaction between chitosan (biopolymer naturally containing primary amine groups), bovine serum albumin (BSA), and gelatin with genipin [73], proposed a mechanism depicted in Figure 5.

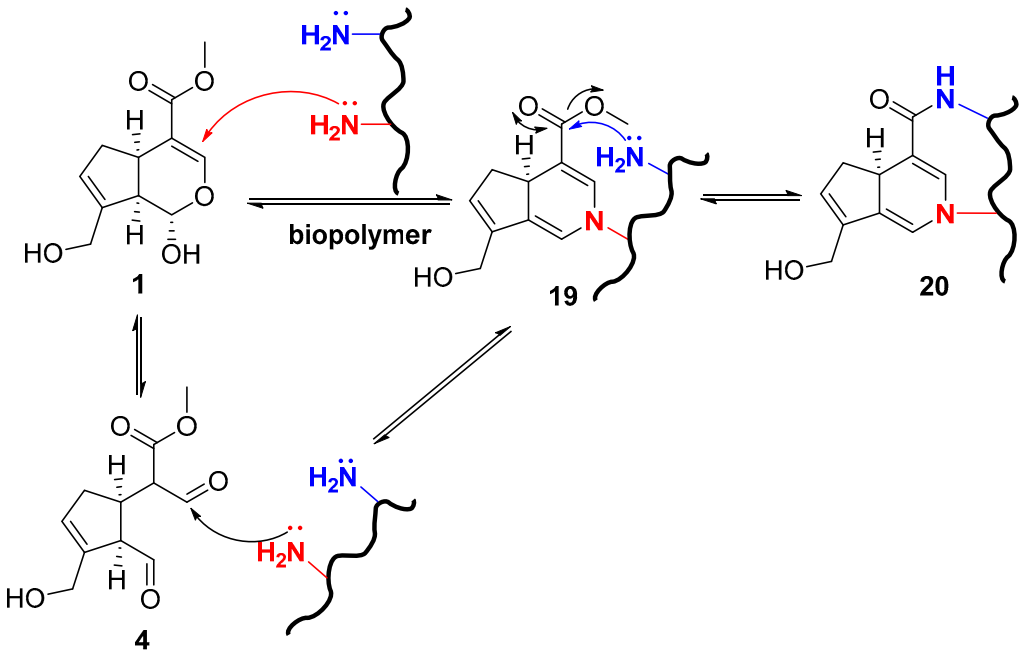

Figure 5. Possible mechanism for the crosslinking of amine-containing biopolymers with genipin, according to Butler et al. [73].

The initial step was suggested to be the nucleophilic attack of one amine group from the biopolymer (in red) to α, β-insaturated ester, with the corresponding opening to yield 19, in a similar way as postulated by Touyama [74] (Figure 5, Mechanism B). In the second step, another amine group from the biopolymer (in blue) will attack the methoxycarbonyl group to produce a secondary amide-type linkage, with the concomitant release of methanol, to produce the crosslinked compound 20. As commented in the section before, according to Di Tomasso et al. [68,69] probably the first step would alternatively proceed via a previous opening of the hemiacetal 1 to the dialdehyde 4, which will be attacked by the amino group in red of the biopolymer to furnish 20.

More recently, Dimida et al. (2015) reported a study of the crosslinking of chitosan with genipin by means of calorimetric analyses (differential scanning calorimetry) and swelling measurements at different pHs and ionic strength, while the reaction kinetics were determined by rheological measurements [75]. These authors supported the cross-linking mechanism depicted in Figure 5, under mild acidic or neutral conditions; some other papers are also bolster this mechanism [76,77].

Another different crosslinking pathway was also considered in the manuscript from Dimida et al. [75], based on the previous work of Fujikawa et al. [71,78], which reported the structure of Genipocyanin G1 (Figure 6, 20), the main product of the reaction between genipin and glycine. These authors did not propose a mechanism for the dimerization, but rather suggested that a radical mechanism might be responsible for the C-C linkage between pentagonal rings of the genipin moieties [78]; thus, they proposed a similar mechanism for yielding the cross-linked structure 21 (Figure 6). This structure has been assumed in the literature [72,75,79–82], although in the original papers from Touyama et al. [74,83], which systematically described the nature of the species produced upon dimerization (or trimerization) of genipin subunits; this type of structure possessing a methylidine linkage between both pentagonal rings was never reported, while some other ones were described.

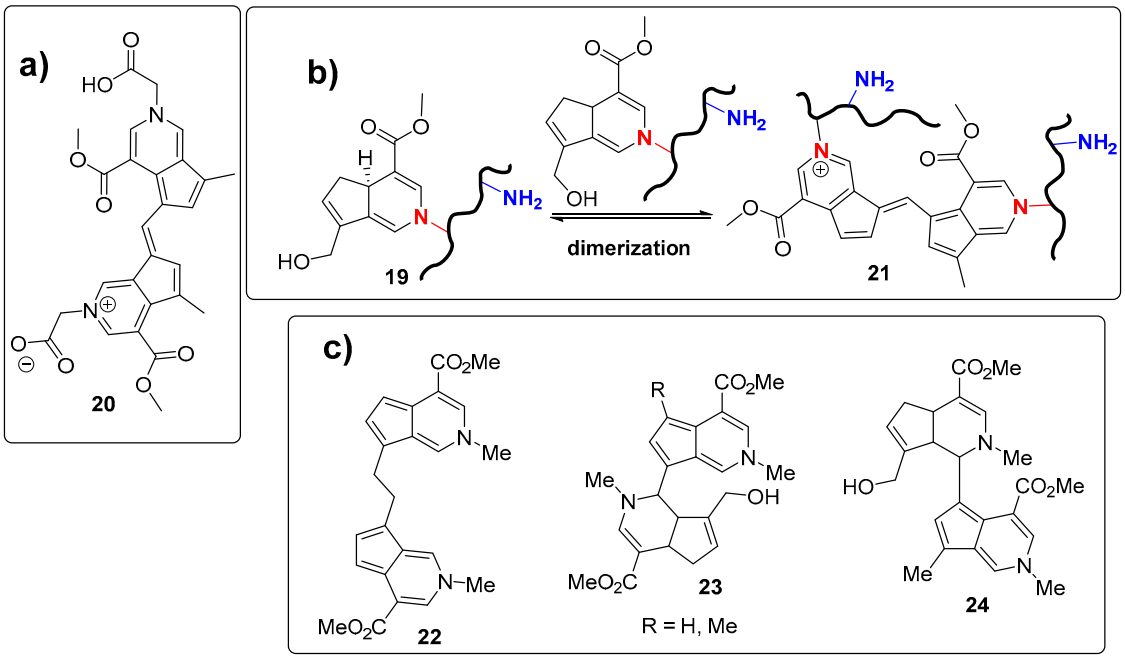

Figure 6. (**a**) Structure of genipocyanin G1, according to Fujikawa et al. [71,78]; (**b**) proposed crosslinked structure; (**c**) dimerization products of genipin, as reported by Touyama et al. [74,83].

It is important to note that, independently from the precise nature of the chemical bonds involved in the crosslinking, genipin is undoubtedly a very useful, non-toxic, and green reagent for the generation of cross-linked biomaterials [67,72,75,77,84–87].

3. Use of Genipin in Biocatalysts Design

Based in the chemistry described above, it is possible to establish how genipin may be used in the preparation of biocatalysts, which kind of reactions will occur (even if they are still under debate) and the groups in support and protein that may become involved in the modification.

3.1. General Aspects of Use of Genipin in Enzyme Immobilization

Genipin has found some interesting uses in enzyme immobilization, mainly due to its low toxicity [88], but also to its good crosslinking features [89]. These pioneering immobilization studies have been performed without really understanding the reaction mechanisms of genipin (see the section above). Thus, it is very likely that the full potential of this reagent is still far from the maximum.

Moreover, it should be considered that in most immobilization cases, when a pre-existing support is used, it tends to be a matrix presenting primary amino groups. That way genipin-activated supports may be considered a heterofunctional support (Figure 7). The first step of the enzyme immobilization on the support may be via covalent immobilization, via anion exchange or via hydrophobic interactions with the layer of genipin. It needs to be considered that the support will be never physically inert, as in the case of using aminated supports and glutaraldehyde [90]. To the best of our knowledge, the versatility of aminated supports activated with genipin has not been adequately exploited, but we have found many different ways of using genipin in enzyme immobilization (activating the support (Figure 8), treating previously immobilized enzymes (Figure 9) or even mixing support, enzyme, and genipin in one step (Figure 10). Next, we present the most relevant papers related to the use of this reagent in enzyme immobilization.

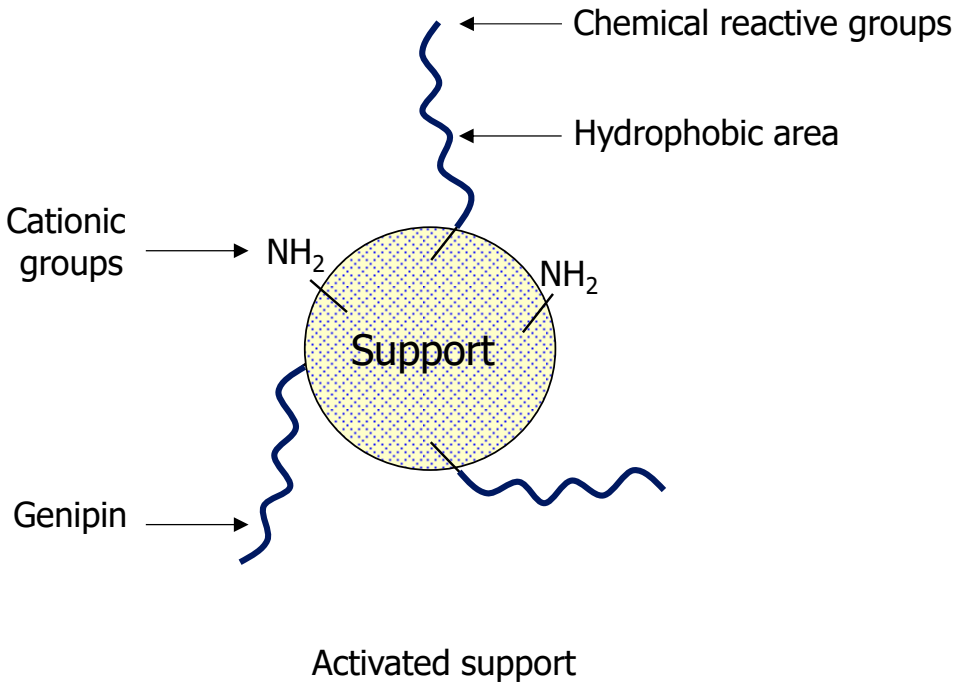

Figure 7. Aminated supports activated with genipin: a heterofunctional support.

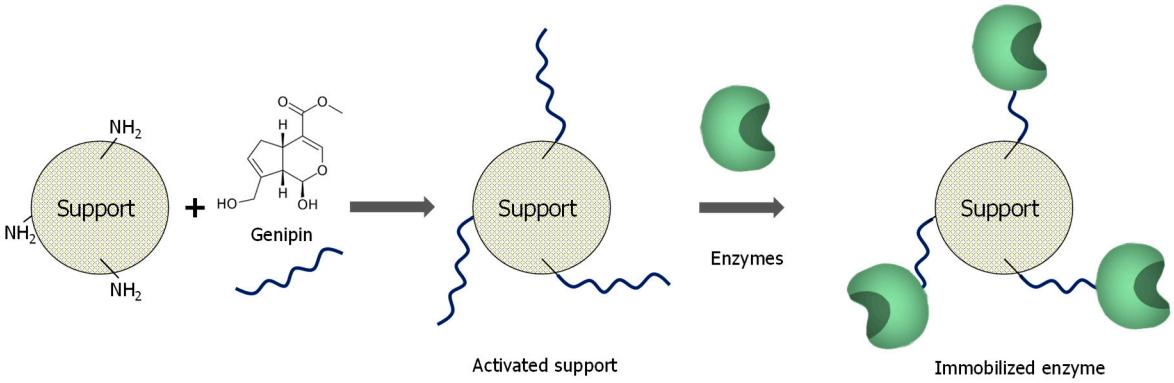

Figure 8. Enzyme immobilization by activation of aminated supports with genipin.

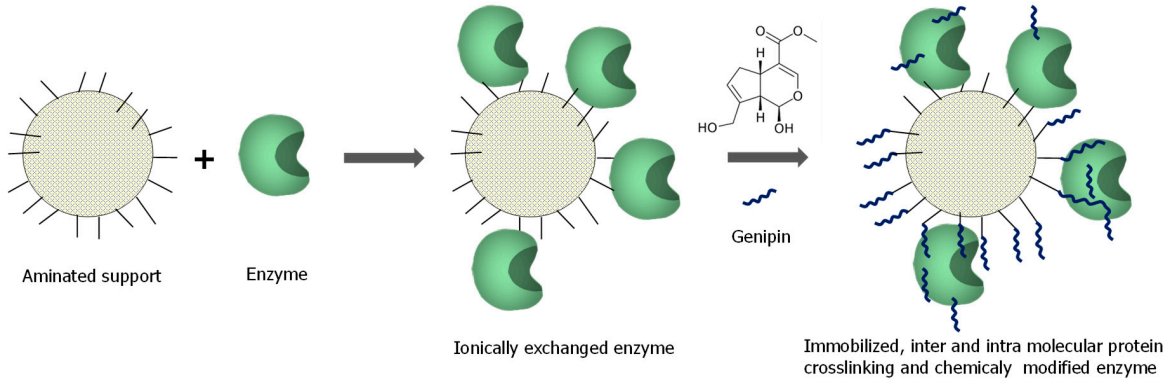

Figure 9. Crosslinking of enzymes immobilized on aminated supports by crosslinking with genipin.

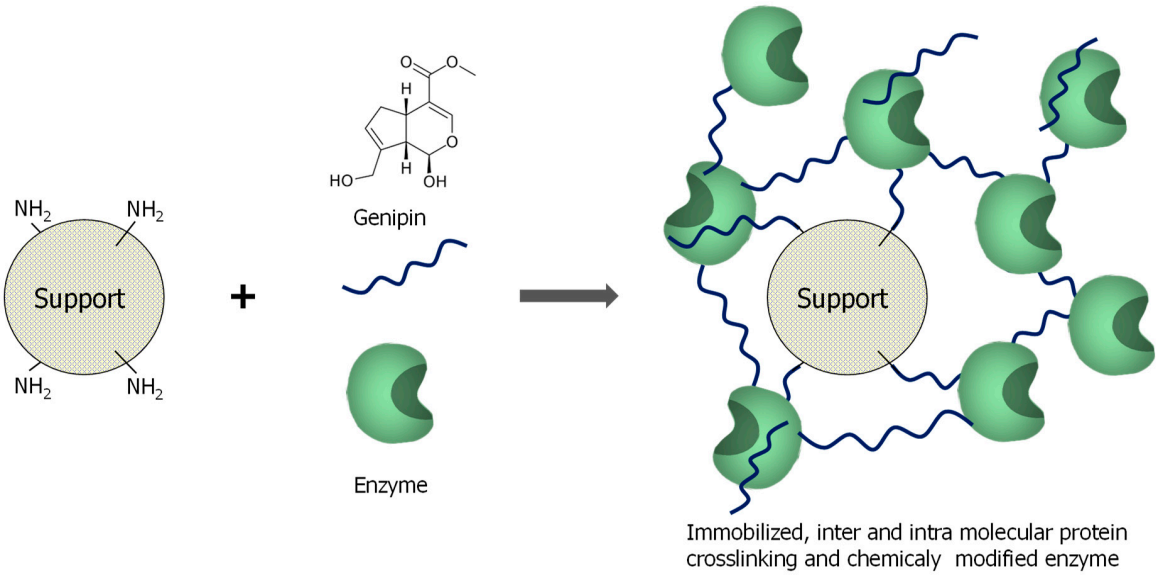

Figure 10. One step immobilization of enzymes on aminated supports using genipin.

3.2. *Enzyme Immobilization on Preexisting Supports Using Genipin*

In an initial work using genipin to prepare an immobilized enzyme biocatalyst, the role of genipin was to crosslink enzymes trapped in alginate and, that way, prevent enzyme release. A β-glucosidase was immobilized on calcium alginate, but the leakage was very high [91]. To prevent this, the trapped enzyme was treated with different crosslinking agents (glutaraldehyde; 1-ethyl-3-(3-dimethylaminopropyl) cardodiimide, *N*-hydroxysuccinimide and genipin). Genipin was the most effective in preventing enzyme leaching, enabling 12 repeated uses without significant activity decrease, while with the other crosslinkers a significant enzyme inactivation could be found [91].

In another research effort, lipase from *Candida* sp. 99-125 was immobilized on two different resins comparing genipin and glutaraldehyde as crosslinker agents [92]. It was found that the immobilized enzyme using genipin exhibited higher storage, pH, and thermal stabilities than the immobilized enzyme using glutaraldehyde. This also resulted in the operational stability being higher using genipin (over 60% of residual activity in the sixth cycle when using genipin while only 35% was attained when using glutaraldehyde) [92]. The same group subsequently reported the immobilization of the same enzyme in a resin, which after immobilization was treated with genipin to obtain covalent attachments (Figure 9). The operational stability of the biocatalyst was quite good, enabling 50 reuses with conversions remaining over 90%.

Chitosan primary amino groups have been used in many instances to immobilize proteins [93], and it has also been used in many cases coupled to genipin to covalently immobilize enzymes (Figure 7). For example, chitosan beads and genipin were utilized to immobilize a lipase [94]. The immobilized enzyme was more active than the free enzyme and resulted in a suitable stabilization at drastic pH values or high temperatures. In another paper, a xylanase was immobilized on magnetic chitosan nanoparticles using genipin to get chitosan-enzyme covalent bonds (apparently mixing genipin, chitosan nanoparticles, and xylanase in one step) [95]. Immobilized xylanase exhibited a higher optimal temperature and broader range of pH where enzyme activity was significantly higher than the free enzyme. However, the operational stability was unsatisfactory [95]. Another publication shows how a purified laccase from *Trametes pubescens* was entrapped in chitosan beads pre-activated with genipin. The immobilization increased enzyme stability under many inactivation conditions, and the resulting biocatalyst decolorized structurally different synthetic dyes without oxidizing mediators [88]. In another research effort, laccase was immobilized on a matrix formed by chitosan crosslinked with genipin and used as a biosensor [96]. The biosensor was utilized in the determination of the total phenolic content of mated herb samples.

Rangel-Rodríguez et al. (2014), reported the crosslinking of chitosan membranes with genipin and used to immobilize fungal pectin esterase. This biocatalyst was successfully used to transform highly-methoxylated pectins into lowly-methoxylated pectins [97]. In another paper, the β-d-galactosidase from *Aspergillus oryzae* was immobilized on chitosan particles followed by treatment with genipin [98]. In this case, the biocatalysts were successfully applied to the production of galacto-oligosaccharides and lactose hydrolysis, maintaining their activity after 25 batches of lactose hydrolysis. The authors classified this biocatalyst as food grade [98].

Using as model the β-galactosidase from *Aspergillus oryzae*, the parameters defining the activation of chitosan has been recently analyzed [89]. The effects of genipin concentration (2.5 mg mL^{-1}), temperature (60 °C), pH (pH 9), and reaction time (1 h) were evaluated in the support activation. The immobilized enzyme was two-fold more stable than the free enzyme, and the authors advance likely structures of genipin in the reaction with chitosan [89].

In some instances, the results achieved in the biocatalyst performance after chitosan activation with genipin were compared with those obtained using other reagents. For example, chitosan beads activated with glutaraldehyde or genipin were used to immobilize a keratinase from *Purpureocillium lilacinum* LPSC #876 [99]. Immobilization yield was similar using both reagents, but expressed activity was higher using genipin. This biocatalyst showed enhanced stability (at drastic pH values or high temperatures) and retained more than 60% of its initial value after five hydrolytic cycles [99]. In another paper, lipase from *Candida rugosa* was immobilized on chitosan encapsulated magnetic nanoparticles using glutaraldehyde or genipin [100]. Genipin permitted to prepare the biocatalysts with the best performance; for example, after five reaction cycles, the biocatalyst prepared using glutaraldehyde exhibited only 26% of its initial activity while the one prepared using genipin maintained over 80%. In fact, this biocatalyst was more stable under drastic pH and temperature, and also presented better operational stabilities than the one prepared using glutaraldehyde [100].

In another paper, commercial β-glucosidase was immobilized by mixing it with chitosan beads and genipin [101]. The biocatalysts beads improved their resistance under acidic conditions after crosslinking. When using the traditional glutaraldehyde immobilization (preactivating the support) (Figures 7 and 8), the activity recovery was lower and the immobilization protocol was longer. The genipin-immobilized enzyme was also more stable than the free or the glutaraldehyde-immobilized enzyme. This biocatalyst was applied to hydrolyze genistin to genistein, maintaining 85% of conversion after 5 cycles [101].

It is important to mention that genipin is not always the reagent that offers the best results. For example, acidic proteases from Monterey sardine stomachs were immobilized onto chitin flakes using genipin or Na-tripolyphosphate with similar (good) performance; the authors reported a great importance on the deacetylation degree of chitosan more than on the chemical reagent [102]. In another example, polyethyleneimine was fixed on a chitin film using epichlorohydrin, glutaraldehyde or genipin [103]. Laccase was then immobilized on the modified film, as polyethylenimine is a very good ion exchanger. In this instance, epichlorohydrin was the crosslinker reagent that gave the best results [24]. Other authors report the immobilization of cytosine arabinoside on bacterial magnetosomes just physically adsorbed or using some crosslinkers, finding that the best loading was achieved using direct adsorption [104].

It should be considered anyway that in these papers, the versatility of glutaraldehyde was not applied to obtain the most active and stable biocatalyst [53,105–109]. The same situation occurs with the genipin, since generally only one condition is used (Figure 7). In that way, it is likely that the genipin potential in enzyme immobilization is still undervalued.

In some other cases, genipin was used in the production of the support. For example, superparamagnetic carboxymethyl chitosan nanospheres were successfully synthesized using genipin as a cross-linking agent of the chitosan [110]. These nano-spheres were then used to immobilize lysozyme.

In other cases, the objective was not to have an irreversibly immobilized enzyme, but to control the way the enzyme is leakage from the support, as enzyme deliverable system. For example, gelatin films were treated using genipin and used to trap lysozyme in a way that they could be released to prevent microbial contamination [111]. Non-crosslinked gelatin films are too unstable, mainly in high humidity environments, for this use. Genipin crosslinked films reduced the lysozyme release at pH 7.0 but not at pH 3.8. On the other hand, antimicrobial activity of the lysozyme-films decreased when genipin concentration increased, indicating too slow an enzyme release. The authors claimed that these films were able to prolong the antimicrobial effects of lysozyme during food storage, increasing food safety [111]. Later, another research group also prepared gelatin films crosslinked with genipin to immobilize lysozyme analyzing the swelling, thermal, water vapor permeability, and tensile property features of the obtained material. Increasing the genipin concentration increased the stiffness, tensile strength, and mechanical resistance of the films. Moreover, the swelling decreased and the stability in aqueous media and at high temperature increased after genipin crosslinking [49]. Gelatin microgels were obtained by cooling and crosslinking with genipin of the dispersed gelatin drops [112]. These gels were used to immobilize a β-galactosidase and this was used to deliver the enzyme. These biocatalysts maintained the activity after being freeze-dried. It was stable at 37 °C at neutral and gastric pH values, indicating the suitability of this biocatalyst for solving lactose intolerance [112].

3.3. Enzyme Immobilization without Support Using Genipin to Stabilize the Solid

In this instance, there is not a pre-existing solid where the enzyme is immobilized, but the solid is formed ex-novo during the immobilization of the enzyme [113]. One advantage of these protocols is that they save on support expenses. However, they have also some disadvantages, as a lack of control of the mechanical resistance, high diffusional problems, and not largely significant enzyme stabilization effects [35] except for multimeric enzymes, as they prevent enzyme subunit dissociation [27,114]. Genipin has been employed in some of these strategies; thanks to its high potential as a crosslinker agent, it can be used to produce intermolecular enzyme crosslinking (Figure 11). For example, cross-linked enzyme aggregates (CLEAs) that are biocatalysts formed by the crosslinking of previously aggregated enzymes (by adding some enzyme precipitant reagent) [115,116]. This immobilization method is very simple and has a great popularity, but one of the critical points is the crosslinking step. That way, CLEAs of a lipase were prepared using polyethyleneimine and genipin to get a good crosslinking [117]. This composite was further coated with silica, using the induction caused by the PEI [24]. Immobilization yield was 88% using this strategy [117]. Later, a purified acid urease from *Providencia rettgeri* JN-B815 was immobilized using the CLEA method using genipin as the crosslinker and bovine serum albumin as the protein feeder [118]. The biocatalyst was applied to remove urea with good reusability (around 80% of the activity was maintained after six reaction cycles). It was applied in rice wine with good elimination of urethane and neither affecting its flavor nor fragrances. The removal rate of urea was still up to 7.56 mg L^{-1} h^{-1} and the biocatalyst was re-used six times [118].

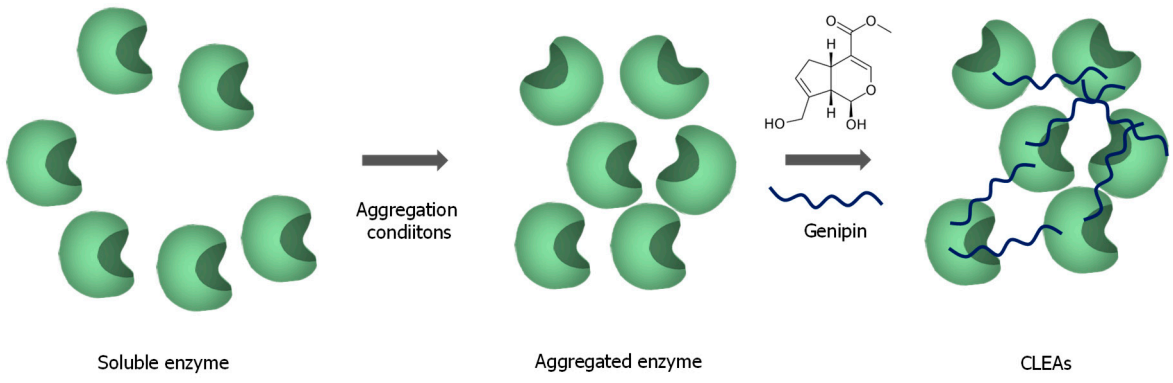

Figure 11. Cross-linked enzyme aggregates (CLEAs) with genipin.

The copolymerization of enzymes is an older immobilization strategy, currently not largely utilized due to the difficulties in having reproducible results. In this strategy, the enzyme is mixed with some polymerizing reagent, which forms a solid involving the enzyme in the polymer formation [119,120]. Genipin has been also used to immobilize enzymes using this strategy (Figure 12). That way, it was used to form the solid biocatalysts particles starting from soluble enzymes. Free glucose oxidase and catalase were co-cross-linked using genipin, producing micro copolymerized enzyme biocatalysts [121]. This permitted to accelerate the reaction of the gluconic acid production and the rapid hydrogen peroxide destruction [122]. The reaction rate increased by a 10% during the whole cycle (perhaps due to the oxygen production by the catalase and the concomitant prevention of oxidase inactivation) [121,122].

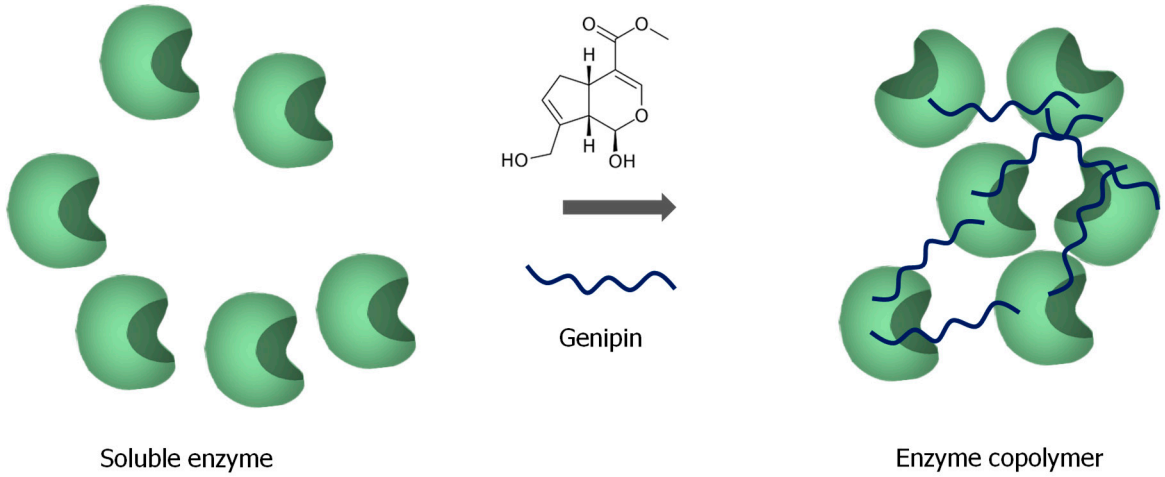

Figure 12. Copolymerized enzyme biocatalyst crosslinked using genipin.

3.4. Immobilization of Cells Using Genipin

Another application of genipin in the production of biocatalysts is the immobilization of whole cells. However, due to the relative short history of this reagent in biocatalysts design, we have been able to find just one example. S-acetylthio-2-methylpropionic acid was produced using immobilized cells of *Pseudomonas fluorescens* IFO 12055, using its lipase activity [123]. Standard trapping of the cells in calcium alginate beads offered unsatisfactory results, while the addition of polyethyleneimine and genipin permitted to obtain a stable biocatalyst. It was used for 25 batches maintaining enantiomeric excesses values higher than 90%.

4. Conclusions

After viewing all the applications of genipin on medical and biocatalysts design application, it can be stated that genipin has proved to be a promising crosslinker, with reduced toxicity. Even when most likely all the possibilities of this compound have not been utilized (the genipin mechanism of action is still under debate, the heterofunctionality of the supports is not used), results are usually comparable or even better that those obtained using other common crosslinkers, like glutaraldehyde. In fact, the prices of geniposide, the starting product, are similar to that of genipin. That means that the enzymatic step to hydrolyze this compound is not determining the price. Its extraction from the natural sources or its chemical synthesis may the main responsible for these high prices. Nowadays, applications in medicine (drug, enzymes, and other compounds controlled delivery, tissue scaffolds, etc.) surpass the applications in biocatalysis, as in these applications toxicity is a major issue and price may be present a lower impact in the final price. That makes reduce the demand or a cheaper genipin. Its general use in biocatalyst design, is hitherto limited by the high commercial price that it has compared to its competitors, as in this applications prices of the reactive are more relevant. It may be expected that when its uses expand in this area, a decrease in price will most likely occur and this can facilitate further expansion of the uses of genipin into biocatalyst designs. Nevertheless, the very good results

reached in these biomedical applications confirm the high potential of genipin as a crosslinker, and those properties are also key in the design of biocatalysts, mainly to be used in food modification. That way, it is expected that the applications of genipin, both at the industrial and academic level, in the area of biocatalyst design will rapidly increase in the coming years.

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