Process Biochemistry 97 (2020) 27–36

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

Modifying alcalase activity and stability by immobilization onto chitosan aiming at the production of bioactive peptides by hydrolysis of tilapia skin gelatin

Kimberle Paiva dos Santos[a], Carolina Mellinger-Silva[b], Ana Iraidy Santa Brígida[b,c], Luciana Rocha Barros Gonçalves[a,*]

[a] Departamento de Engenharia Química, Universidade Federal do Ceará, Campus do Pici, Bloco 709, CEP 60455-760 Fortaleza, CE, Brazil
[b] Embrapa Agroindústria de Alimentos, Av. das Américas, 29501 – Bairro Guaratiba, CEP 23020-470, Rio de Janeiro, RJ, Brazil
[c] Embrapa Agroindústria Tropical, R. Dra Sara Mesquita, 2270 – Bairro Planalto do Pici, CEP 60511-110, Fortaleza, CE, Brazil

ARTICLE INFO

Keywords:
Alcalase
Chitosan
Immobilization
Stability
Protein hydrolysates
Tilapia skin gelatin

ABSTRACT

The protease from *Bacillus licheniformis*, commercially known as Alcalase®, was insolubilized and stabilized by immobilization onto activated chitosan. Activation with different agents, such as glutaraldehyde (GLU-Chi), glyoxyl (GLY-Chi) and divinyl sulfone (DVS-Chi) was investigated. The effect of the immobilization protocol, for instance different pH and times, were also evaluated. GLU-Chi showed the highest activity (35.6U_{NPA}/g) with the smallest substrate (N-Boc-L-alanine p-nitrophenyl-ester, NPA), while GLY-Chi showed the highest activity (1.5 $U_{Azocasein}$/g) using the greatest substrate (azocasein). A 24-h immobilization period was enough to stabilize the enzyme using the three supports under almost all conditions. Operational stability in azocasein hydrolysis was assayed and GLU-Chi showed no activity loss during 5 cycles. DVS-Chi retained around 70 % of its initial activity after the fifth cycle, whereas GLY-Chi activity retained only 10 %. Finally, the biocatalysts were used in the hydrolysis of tilapia skin gelatin aiming the production of peptides with antioxidant activity. The protein hydrolysates obtained using GLU-Chi presented the highest antioxidant activity (36.7 μM Trolox Eq). However, the best results of operational stability were obtained using DVS-Chi, which did not lose its initial activity after 3 consecutive cycles of gelatin hydrolysis.

1. Introduction

Alcalase®, a protease from *Bacillus licheniformis*, is an useful enzyme with broad application in several industries and may be used in chemoenzymatic synthesis, detergent formulations and in the production of protein hydrolysates containing bioactive peptides [1,2]. This enzyme is commercially distributed as an extract containing several proteolytic enzymes and its major component is subtilisin Carlsberg, an enzyme of broad specificity with a 27.3 kDa molecular weight [3,4].

The consumption of healthiest food has been more required by consumers, and this industry has sought for components containing such enhanced properties [5]. Alcalase has been used in the synthesis of protein hydrolysates from several sources, as goat whey [4], soy [6], chickpea [7] and fish [8], aiming at the production of bioactive peptides. Several studies involving the use of soluble alcalase in fish gelatin hydrolysis with promising results have been performed [8–10]. However, the use of proteases in its soluble form may represent a disadvantage to the process. In addition to the instability problems common to other enzymes, proteases can undergo autolysis, accelerating the deactivation rate [11], reducing reaction yield ($g_{Product} \cdot g^{-1}_{Reactant}$) and effective use of the biocatalyst ($g_{Product} \cdot g^{-1}_{Biocatalyst}$). Enzyme immobilization is a strategy to solve some of these hindrances related to the industrial use of these biocatalyst [12,13], and it can be achieved by using different methodologies.

Immobilization by covalent binding, for instance, is the mechanism that has provided the highest enzyme stabilization due to the stability of the bonds formed between support and enzyme [14]. Particularly, the multipoint covalent attachment is one of the most studied strategy to attain high enzyme stabilization due to the obtained enzyme structure rigidification, which prevents the disarrangement of the tertiary structure [15,16]. In addition, the conditions used in an immobilization protocol may define the orientation of the immobilized enzyme, which

* Corresponding author.
E-mail addresses: kimberleps@gmail.com (P.d.S. Kimberle), caroline.mellinger@embrapa.br (M.-S. Carolina), ana.iraidy@embrapa.br (I.S.B. Ana), lrg@ufc.br (R.B.G. Luciana).

https://doi.org/10.1016/j.procbio.2020.06.019
Received 18 April 2020; Received in revised form 22 June 2020; Accepted 23 June 2020
Available online 24 June 2020
1359-5113/ © 2020 Elsevier Ltd. All rights reserved.

is a crucial point for proteases used in protein hydrolysis because of the diffusional limitations related to the hydrolysis of its substrates [17].

In this work, alcalase has been covalently immobilized onto chitosan, a natural, renewable, biodegradable and low-cost organic polymer derived from chitin. It is often used as support for the immobilization of different enzymes due to presence on its surface of two functional groups, amino ($-NH_3$) and hydroxyl ($-OH$) [14,18,19], that can be easily modified to form covalent bonds with the enzyme molecules. In order to establish multipoint covalent bonds for enzyme stabilization, glutaraldehyde, glyoxyl and vinylsulfone activated supports are among the most adequate immobilization strategies [20].

Glutaraldehyde may interact with the enzyme by different ways because it contains ionic and hydrophobic moieties [21,22]. Furthermore, a first enzyme-support approximation may occur by ionic interactions when supports (such as chitosan) have primary amino groups. The literature reports that if the concentration of glutaraldehyde used in the support activation is higher than what is considered moderate (a moderated condition is achieved by using a concentration between 0.1 %–1 % v/v, at pH 7 for 1 h), two molecules of glutaraldehyde per amino group may be obtained. Since this dimeric form (two glutaraldehyde molecules/amino group in the support) is considered the most reactive form versus amino group in the enzyme, in this work, a glutaraldehyde concentration of 5% was employed for support activation [23]. Glutaraldehyde in the support may react with different regions of the enzyme, but usually with residues containing primary amino groups, such as lysine, arginine, asparagine, glutamine and the terminal amino [11,23,24]. Fig. 1A shows alcalase's surface and the residues that may form Schiff bases with glutaraldehyde.

Another modified chitosan support can be obtained by the activation of its hydroxyl groups using glycidol and sodium periodate to form aldehyde-glyoxyl groups [25]. Glyoxyl supports are able to establish intense covalent attachments (Schiff base) with amino groups of lysine or terminal amino in the enzyme (see Fig. 1B), what makes it widely employed for enzyme stabilization [26,27]. Relevant results on alcalase stabilization as well as on immobilization for application in protein hydrolysates production have been reported so far [2,28]. Thus, this strategy was chosen for comparison reasons.

Last but not least, divinyl sulfone (DVS) is another bifunctional that can present a heterofunctional behavior depending on the material used for the modification [29]. DVS acts similarly to glutaraldehyde and may modify both amino and hydroxyl groups present on the chitosan surface [15]. The Vinyl sulfone functional group may react with several groups — primary or secondary amino, hydroxyl, imidazole or thiol. Moreover, DVS supports shows higher reactivities towards lysine, tyrosine and histidine (which are present in alcalase surface, see Fig. 1C) and cysteine residues, depending on the pH conditions. At pH 10, the highest reactivities are exhibited for lysine, cysteine and histidine, whereas at pH 7, a moderate reactivity is observed with cysteine and histidine [30–32]. The covalent bonds using DVS-supports can be even more intense than with glyoxyl supports [31,32]. Similarly to the other supports, the orientation of the immobilized enzyme onto DVS-supports can also be turned by using different pH conditions, since the pH range used for this support can be from pH 5 to pH 10 [30].

In this work, hydroxyl and amino groups on chitosan were activated by different agents - glutaraldehyde, divinyl sulfone and glyoxyl - aiming at the establishment of covalent bonds with alcalase. The influence of immobilization time and pH conditions on the immobilization rate and on the properties of the obtained biocatalysts was analyzed. Firstly, the influence of pH on the rate and yield of immobilization was studied and an overview of the structure and reactivity of the enzyme residues was correlated with the results. Secondly, the effect of immobilization time, under different pH values, on the thermal stability was studied. In addition, an evaluation of the biocatalyst specificity, using a small and a large substrate, as well as its operational stability, was performed. Finally, the biocatalysts with the best results of thermal and operational stability were used to catalyse the hydrolysis of tilapia skin gelatin in order to produce peptides with antioxidant properties.

2. Experimental

2.1. Materials

Alcalase, a Protease from *Bacillus licheniformis* (declared activity 2.4 U/g), was purchased from Sigma-Aldrich (St. Louis, USA). Powdered chitosan (low molecular weight), NPA (N-Boc-L-alanine p-nitrophenyl-ester), Glycidol, Divinylsulfone, Glutaraldehyde, DPPH (2,2-diphenyl-1-picrylhydrazyl), Trolox and azocasein were also purchased from Sigma-Aldrich. All other chemicals used were of analytical grade.

2.2. Preparation and modification of support for enzyme immobilization

Chitosan gel was prepared through a procedure based on the methods of two works described in literature with a slight modification [25,33]. 10 g of powdered chitosan were initially dissolved in 240 mL of an aqueous 5 % (v/v) acetic acid solution. The obtained solution was added to 250 mL of a 1 M NaOH solution and gently stirred for 24 h, at room temperature. Afterwards, the chitosan gel was washed with an excess of distilled water and we performed a vacuum filtration.

2.2.1. Glutaraldehyde activation

The glutaraldehyde activation was conducted according to the methodology described in literature with some modifications [25]. 1 g of chitosan gel was suspended in 9 mL of 100 mM sodium phosphate buffer, pH 7.0, containing 5 % (v/v) of glutaraldehyde, and the mixture was kept under continuous stirring for 1 h, at room temperature. Afterwards, the activated support was washed with an excess of distilled water.

2.2.2. Glyoxyl activation

The preparation of the glyoxyl-chitosan was carried out according to the standard protocol described in literature [34]. 1 g of chitosan gel was added to 3 mL of an aqueous solution (1.7 M NaOH and 0.75 M $NaBH_4$) using an ice/water cold bath. Afterwards, 0.48 mL of glycidol was added and the solution was kept under gentle stirring for 18 h. Then, this modified chitosan was washed with an excess of distilled water. After, the modified chitosan was incubated in 2 mL of a 0.1 M $NaIO_4$ solution and kept under stirring for 2 h and then the glyoxyl-chitosan was washed with an excess of distilled water and stored at 4 °C.

2.2.3. Divinylsulfone activation

The divinylsulfone activated chitosan was prepared according to literature [29], with minor modifications. 10 g of chitosan gel was added to 200 mL of a 333 mM sodium carbonate solution at pH 10. Later, 7.5 mL of divinylfulfone was added and the solution was stirred for 35 min, at room temperature. The DVS-activated support was washed with an excess of distilled water and stored at 4 °C.

2.3. Enzyme immobilization

Alcalase was immobilized onto activated chitosan, at 25 °C, at different pH values (7 and 10) and immobilization times (from 15 h to 96 h). Alcalase immobilization onto chitosan activated with glutaraldehyde and divinylsulfone was carried out in 100 mM sodium phosphate buffer (pH 7) or in 100 mM sodium carbonate-bicarbonate buffer (pH 10). For GLY-Chi, 100 mM sodium carbonate-bicarbonate buffer at pH 10 was used. The same enzyme load was used in all essays, 100 $U_{NPA}/g_{support}$ and 8 $mL_{solution}/1g_{support}$, which corresponded to 2 $mg/g_{support}$. After the immobilization, the remaining reactive groups of GLU-Chi and DVS-Chi were blocked according to the literature [30], with some modifications. After immobilization onto GLY-Chi, sodium borohydride (0.5 mg/mL) was added to the immobilization medium

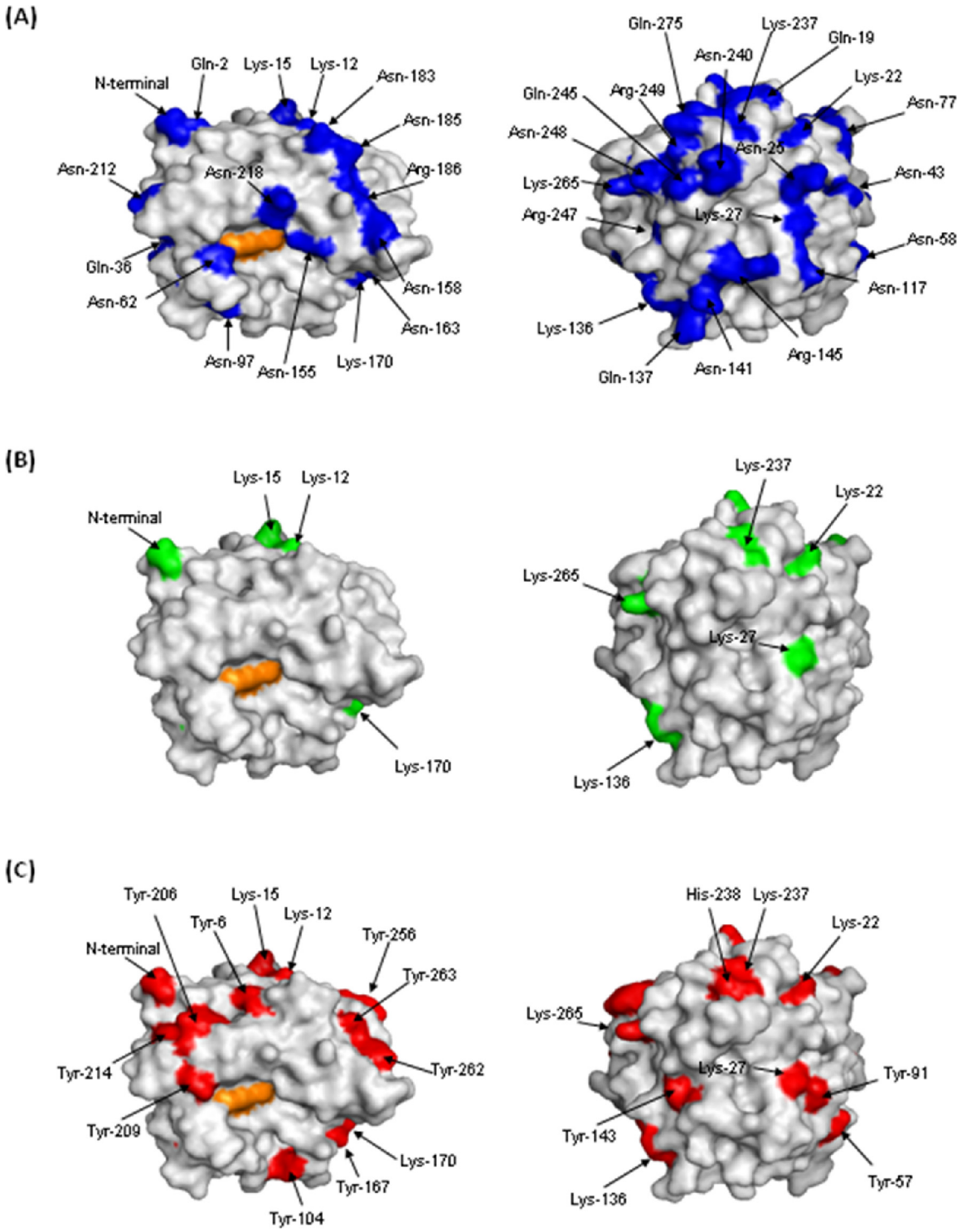

Fig. 1. Schematic representation of the major component structure of Alcalase (subtilisin Carlsberg- Protease from *Bacillus licheniformis*, PDB code 1SBC). Active site (orange): Asp-32, His-64 and Ser-221). Residues that can react with Glutaraldehyde support (A), with Glyoxyl supports (B) and with DVS supports (C).

and the mixture was kept under stirring for 30 min at 25 °C. After this, the immobilized enzyme was washed with an excess of distilled water.

2.4. Determination of enzyme activity by NPA hydrolysis

The determination of enzyme activity was carried out by using the substrate Boc-Ala-Onp (NPA), according to the methodology presented by [27], with minor modifications. The hydrolysis of the substrate (100 mM NPA, prepared in acetonitrile) was conducted at pH 7 (50 mM sodium phosphate buffer, containing 20 % of ethanol) and 25 °C. The product formation was quantified in a spectrophotometer (405 nm) and the enzyme activity was expressed in U_{NPA} ($1U_{NPA}$ = amount of enzyme that was capable of hydrolysing 1 μmol of substrate per minute at pH 7 and 25 °C).

2.5. Determination of enzyme activity by azocasein hydrolysis

The reaction was carried out according to literature [35], with slight

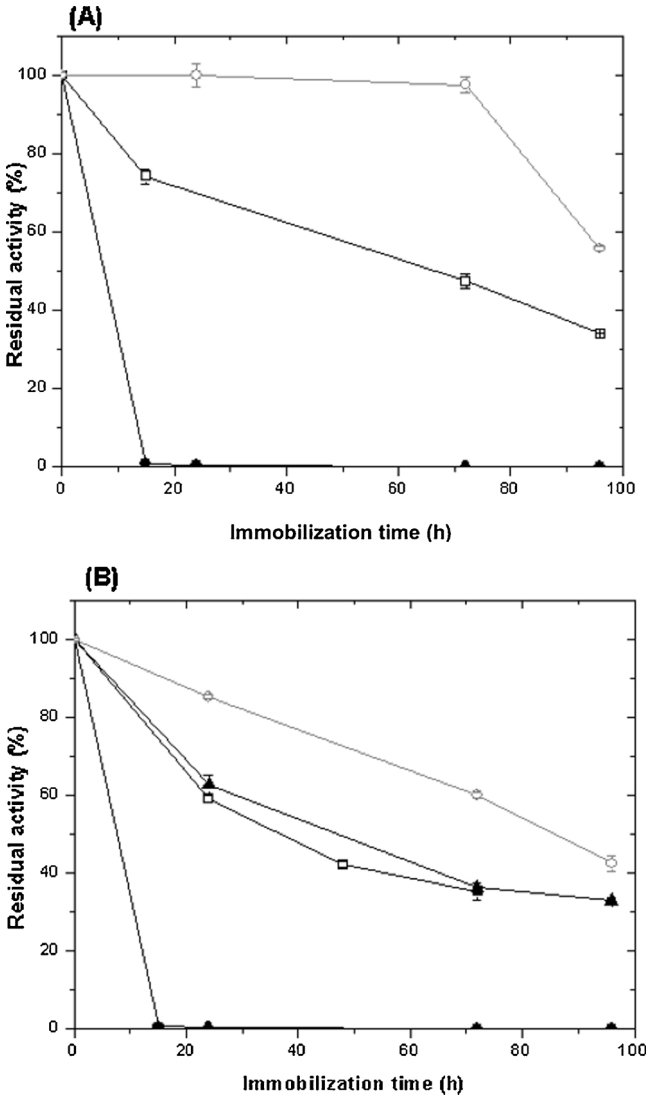

Fig. 2. Immobilization course at pH 7 (A) and 10 (B). Free enzyme (○). Supports GLU-Chi (●), DVS-Chi (□) and GLY-Chi (▲).

modifications. 2 mL of an 0.5 % azocasein solution, prepared in 50 mM sodium acetate buffer (pH 5), was mixed with 2 mL of sodium acetate buffer, and, then, the biocatalyst mass was added. The reaction was conducted at 32 °C and 120 rpm, for 40 min. The biocatalyst activity was quantified by analyzing the supernatant at the end of the reaction. The supernatant solution was added to a 15 % (w/v) trichloroacetic acid solution to precipitate the molecules of the remaining unhydrolyzed proteins. After the precipitation, the mixture was centrifuged at 3000 rpm, for 15 min. Finally, the supernatant was added to a 5 N potassium hydroxide, and the absorbance, at 428 nm, of this solution was measured. It was established that 1 unit of proteolytic activity ($U_{azocasein}$) was correspondent to the enzyme amount which produces a 0.01 increase in the measured absorbance.

2.6. Thermal stability

The thermal stability of the free and immobilized alcalase was studied by incubating the biocatalysts in a 100 mM sodium phosphate buffer, at pH 8 [27] and 60 °C. For the free, a dialyzed extract (against distilled water, at 4 °C, with periodic water changes for 6 days), which was free of stabilizers like the immobilized enzyme, was used in the assay. Thus, it was possible to analyze the real effect of the immobilization technique on enzyme stability. Periodically, samples were withdrawn, and the activity was quantified by the NPA hydrolysis. The half-life ($t_{1/2}$) values were calculated by using the model proposed by Sadana and Henley [36].

2.7. Operational stability

In order to analyze the operational stability of the immobilized enzymes, consecutive cycles of azocasein hydrolysis were performed (as described in Section 2.5) using these immobilized enzymes as biocatalysts. After each cycle, the biocatalyst mass was separated, washed with an excess of distilled water and dried by vacuum filtration. The biocatalyst mass was weighted and applied in a new azocasein hydrolysis cycle.

2.8. Bioactive peptides production by tilapia skin gelatin hydrolysis

The tilapia skin gelatin hydrolysis was performed with immobilized or free alcalase as a biocatalyst according to literature [37], with minor modifications. The reaction was conducted at 55 °C, gelatin 1% (w/v), and pH 6, for 2 h, under constant stirring. The added mass of immobilized biocatalyst was equivalent to $1U_{NPA}$ per mL of reaction solution or enzyme/substrate ratio of 5% for free alcalase. At the end of the reaction cycle, the insoluble biocatalyst was removed from the solution, which was then submitted to the bifunctional activity analysis of the peptides produced by the protein hydrolysis. The biofunctionality analyzed was that of antioxidant capacity according to literature [38], by determining the scavenging effect of the produced peptides on the DPPH free radical using a 0.1 mM DPPH solution prepared in ethanol. Afterwards, 1.5 mL of this solution was added to 1.5 mL of the final hydrolysis solution in a test-tube and mixed in vortex for 10 s. After 30 min of resting at 25 °C, the absorbance was measured in spectrophotometer at 517 nm. The activity was calculated using a Trolox standard curve and expressed as μM Trolox Equivalent. The peptide profiles in the hydrolysate was determined by high performance liquid chromatography in reverse phase (RP-HPLC), performed in a high-performance chromatograph (Jasco - Tokyo, Japan)), equipped with an ultraviolet (UV) detector and Jasco ChromNAV software. The method uses a Hypersil BDS C18 (Thermo) column at a flow rate of 1 mL/min, held at 30 °C. Detection was at 216 nm. Total running time was 40 min. The mobile phase was programmed in a gradient (5–95 %, v/v) of acetonitrile in ultrapure water, containing 0.1 % trifluoroacetic acid (TFA) in both solutions. Tilapia skin gelatin solution at 1 % (w/v) was used as standard.

3. Results and discussion

3.1. Immobilization course of alcalase onto chitosan-based supports at different pH values

First, the influence of the activation method on the immobilization rate was investigated at different pH values. For this reason, alcalase was immobilized onto chitosan functionalized with different reactive groups: glutaraldehyde (GLU-Chi), glyoxyl (GLY-Chi) and divinyl sulfone (DVS-Chi). In order achieve the immobilization via a direct first covalent attachment, a moderate ionic strength was used (100 mM buffer), to avoid the ionic exchange between the support and the enzyme [23]. Fig. 2 shows the immobilization course at pH 7 and 10, in which one can see that immobilization onto GLU-Chi was faster when compared to the other supports, regardless of pH value. Immobilization onto GLY-Chi (only at pH 10, Fig. 2 B), on the other hand, was very slow, since residual activity in the supernatant was 32.85 % even after 96 h.

Support functionalization with divinyl sulfone involves both hydroxyl and amino groups present on the surface of chitosan [29,39]. Therefore, the amount of available bindings sites on the DVS-Chi for enzyme immobilization should not be lower than in GLU-Chi at any pH.

A possible explanation lies on the high reactivity of divinyl sulfone, which may generate a crosslinking between some of their own molecules, which is not a desirable reaction. This undesirable crosslinking may restrain the formation of a higher amount of binding between the enzyme molecule and the support [22,29].

Moreover, that behavior, a faster immobilization rate onto GLU-Chi compared to the DVS-Chi and GLY-Chi, was expected and can be explained by analyzing Fig. 1. There are much more residues in alcalase able to react with a GLU-Chi than with DVS-Chi or GLY-Chi, which allows this faster immobilization. It can be observed that the moieties in alcalase able to react with GLU-Chi are numerous and well dispersed in the molecule surface, which can also allow different orientations of the enzyme during immobilization, depending on pH used. Regarding GLY-Chi, alcalase presents a low content of lysine residues, able to react with this support. Moreover, they are quite dispersed, which may hinder the establishment of covalent interactions with this support that should be achieved via areas rich in the reactive residues [32]. Using DVS-Chi, the enzyme presents a moderate number of residues able to interact, which are more reactive at pH 10 than at pH 7, as introduced before, which promotes a faster immobilization at the alkaline pH.

3.2. Effect of immobilization time on the thermal stability of the insoluble enzymes

Some critical variables should be considered when evaluating the immobilization of an enzyme. In addition to the rate and yield of immobilization, stability also deserves attention [40] and temperature is one of the main parameters that affects the stability of an enzyme. Thermal stabilities, at 60 °C and pH 8, of both free and immobilized enzymes were studied by measuring residual activity, using NPA as substrate, see Fig. 3.

Fig. 4 shows the values of half-life prepared using different immobilization times and pH 7 or pH 10. These values were calculated from experimental data of thermal stability at 60 °C, using the thermal inactivation model proposed by Sadana and Henley [36]. Initially, an increase in the half-life time with the increase of time is observed, but there is an optimal value. Other authors also report similar enzyme stabilization against immobilization time, when immobilizing Alcalase [27] and Novo-Pro D [41], both of them proteases from *Bacillus licheniforms*. It is expected that more enzyme-support bonds are formed at higher contact times, producing enzyme stabilization. When multipoint immobilization occurs, although the first binding is fast, the multi-interaction process requires longer times to achieve the correct alignment of the reactive groups of enzyme and support [42]. After a certain time, the formation of additional covalent bonds can become prejudicial to the enzyme [43], which resulted in the decrease of the enzyme stability observed. Literature reports that an excess of rigidification of the enzyme molecule, as well as certain structural tension caused by immobilization via multipoint covalent attachment, can destabilize the tertiary structure [44–46]. Other authors, which immobilized a protease onto glyoxyl-agarose, reported a similar negative effect on enzyme stability, which could be associated to the conformational changes that resulted from the exposition of some key groups of the enzyme to the medium [47].

For the free enzyme, $t_{1/2}$ = 20.5 min, which is lower than $t_{1/2}$ of all the insoluble biocatalysts. This result proves that almost all immobilized preparations were more stable than the soluble enzyme (more than 4-fold when alcalase was immobilized onto GLU-Chi) and that immobilization conferred to the enzyme a higher stability against thermal deactivation.

In general, higher stabilization was achieved by using GLU-Chi, which was expected by analyzing the representation in Fig. 1. Alcalase demonstrates a higher possibility of multipoint attachment with the glutaraldehyde activated support, because there are several residues very close to each other. Among all the biocatalysts GLU-Chi, it is possible to see (Fig. 4) that immobilization at pH 7 allows a higher

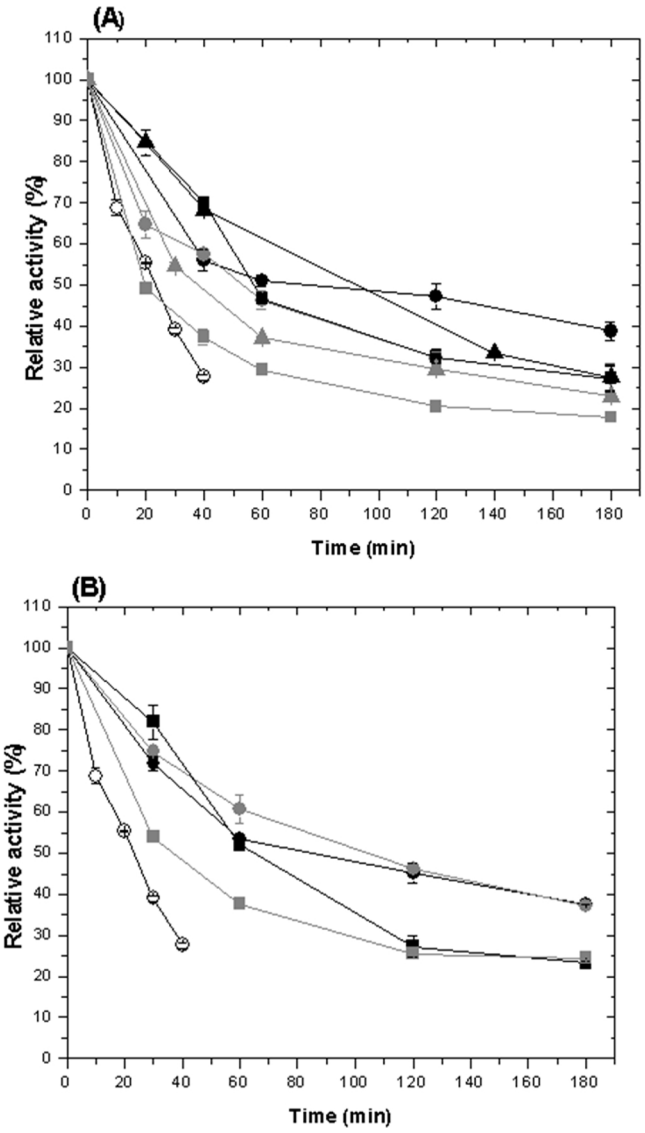

Fig. 3. Thermal deactivation (60 °C, pH 8) profile of the soluble enzyme (○) and the biocatalysts obtained by immobilization at pH 7 (A) and pH 10 (B), for 24 h (black lines) or 72 h (gray lines), on the supports GLU-Chi (●), DVS-Chi (■) and GLY-Chi (▲).

stabilization when compared to the results achieved at pH 10. Since amino residues in the protein were more reactive at alkaline pH [23], higher enzyme stability was expected due to multipoint immobilization. Nevertheless, this further stabilization was not observed and it may have been caused by a number of factors, since immobilization of the enzyme onto the support depends on a delicate balance [48], which is due not only on the support surface but also on the presence of functional groups on proteins and immobilization conditions. Besides that, is has been showed that glutaraldehyde is quite unstable under basic conditions, being able to undergo intramolecular conformation changes, which alters the covalent bonds established with the amino groups of the enzyme [23].

Other authors also observed that stabilization depended on the crosslinker reagent [27]. For instance, Alcalase immobilized on glutaraldehyde-agarose could be stabilized by 6.5-fold, which is similar to the results achieved by alcalase immobilization on GLU-Chi. On the other hand, when the same enzyme was immobilized on glyoxyl-agarose, a 100 – 500-fold stabilization was achieved depending on the immobilization conditions (contact time). This stabilization factor is much higher than that obtained in the current study. It is important to

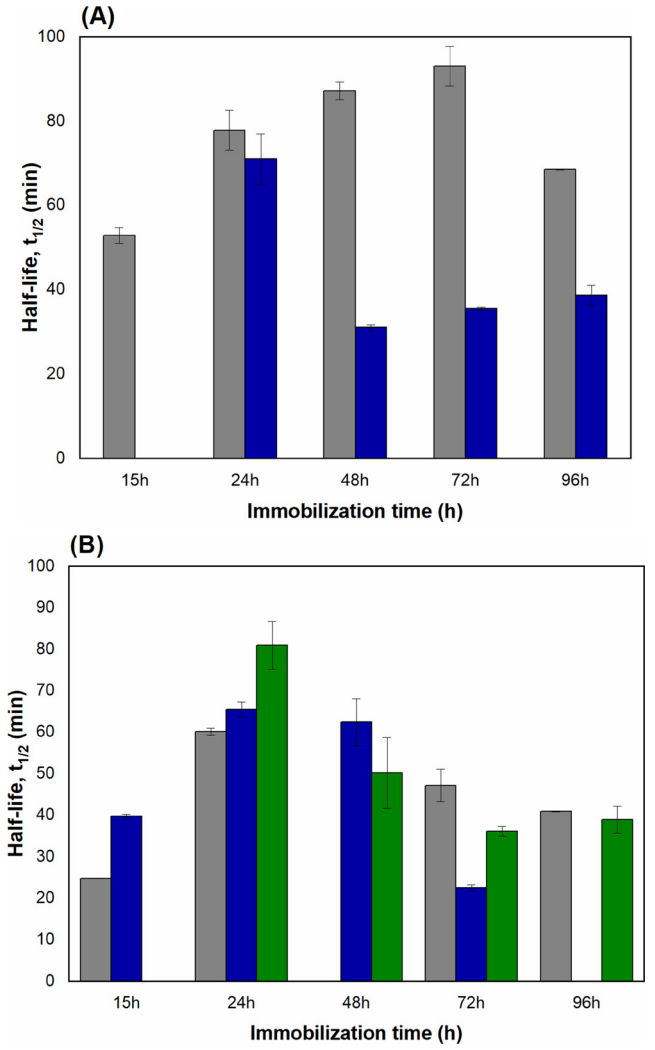

Fig. 4. Thermal stability (60 °C, pH 8), expressed in half-life values, of the biocatalysts obtained by immobilization at pH 7 (A) and pH 10 (B) on the supports GLU-Chi (grey bars), DVS-Chi (blue bars) and GLY-Chi (green bars). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

mention that chitosan and agarose physical properties are quite different. Agarose, a very popular commercial support for enzyme immobilization, presents several advantages that allow enzyme stabilization. The presence of pores in agarose allows a geometrical connection between the protein and the support surface, which is favorable for enzyme immobilization [34]. But, the most important feature may be the weak and reversible imino bound, which requires the establishment of several enzyme-support linkages. Although it may seem a drawback, this feature drives the immobilization through the richest area in reactive groups of the protein, allowing high stability [49]. Probably, the higher degree of activation and/or the protein interaction with the flat surfaces of agarose (inside the pores) may explain the differences in stabilization when compared to chitosan. On the other hand, chitosan can be a cost-effective alternative since it is an abundant and cheap raw material, available in Ceará State, Brazil.

3.3. Biocatalyst activities

Several biocatalysts were obtained from the immobilization onto different supports, at both pH, during different times. Their catalytic activities using NPA, a small synthetic substrate, and azocasein, a large substrate, were determined and the results are shown in Fig. 5. It is

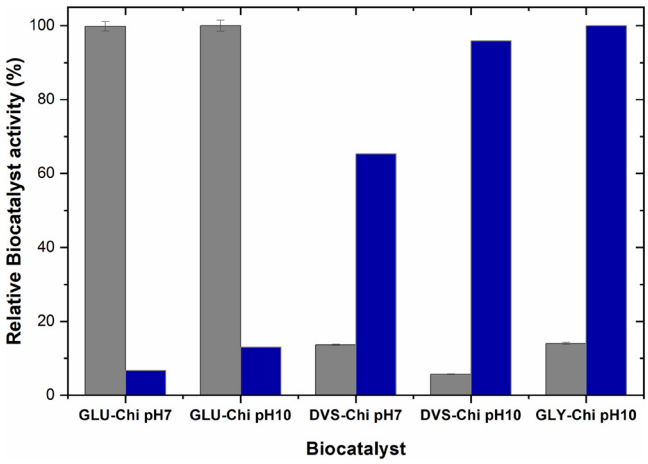

Fig. 5. Effect of immobilization conditions on the activity of immobilized alcalase against NPA (grey bars) and azocasein (blue bars). Relative activity was calculated considering as 100 % the highest value of the activity, 35.6 U_{NPA}/g for NPA and 1.5 $U_{Azocasein}$/g for azocasein. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

possible to see that glutaraldehyde activated preparations presented higher activity vs. NPA, when compared to azocasein. DVS and glycidol activated preparations, on the other hand, were more active against azocasein. As reported, immobilized enzyme activity and selectivity depends on enzyme orientation, as well as on the intensity of enzyme-support reaction, that may promote distortion on different areas of the protein and alter its catalytic properties [22,24,50]. Moreover, using large proteins as substrate, it is important to remark that the access of the substrate to the active center may be determined by steric hindrances originated by that enzyme orientation to the support surface [51].

In addition, by analyzing Fig. 1A, there are three residues (Asn-62, Asn-155 and Asn-218), which are able to be attached to GLU-Chi, which are very close to the active center. If a fraction of the total enzyme molecules are attached to the support via one of these three residues, steric hindrances will hinder the access of a greater substrate, what will decrease the total activity of the immobilized enzyme molecules. Therefore, we can conclude that when alcalase is immobilized onto GLU-Chi, the enzyme active center may not be fully available for the substrate, and the systems may be subject to diffusion limitations and steric hindrance, which explains the best results obtained with a small substrate.

It was observed also that the enzyme activity versus azocasein was slightly higher using alcalase immobilized on to DVS-Chi at pH 10 when compared to the biocatalyst prepared at pH 7. Pointing out, a proper orientation achieved at pH 10, or a different enzyme-support interaction may explain this result. By comparing stability and activity results, enzyme immobilized at pH 10 is both more active and stable.

3.4. Operational stability at 32 °C

The reusability and operational stability of a biocatalyst obtained from an immobilization process is an important characteristic to be studied since it is a key factor for their commercial applications in industry. In order to investigate the operational stability and reusability, preparations that presented the best results of activity and stability were evaluated as biocatalysts for successive cycles of hydrolysis of azocasein. After each run, the biocatalysts were washed with an excess of distilled water, in order to remove the rest of the reaction medium, dried and weighed before the next cycle. Fig. 6 shows the reusability results of those biocatalysts. Alcalase immobilized onto GLY-Chi was almost fully deactivated after 5 cycles while alcalase immobilized onto

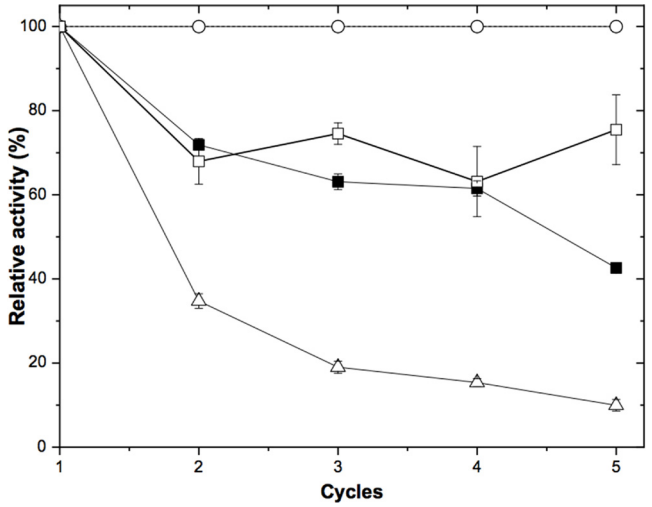

Fig. 6. Operational stability in the azocasein hydrolysis of different Alcalase preparations at 32 °C and pH 5: at pH 7 (closed symbols) and pH 10 (open symbols) onto GLU-Chi (circles), DVS-Chi (squares) and GLY-Chi (triangles).

GLU-Chi was fully stable and alcalase immobilized onto DVS-Chi retained almost 60 % of its initial activity. Enzyme leakage from GLY-Chi is not a possible explanation due to the type of bond formed between the enzyme and the support (secondary amino groups) [52]. Therefore, this biocatalyst is not the best option for those long-term reaction conditions.

3.5. Bioactive peptides production

Soluble alcalase was used to produce tilapia skin gelatin hydrolysate and, after 3 h of reaction, 77 % of its initial total protein was hydrolyzed in peptides smaller than 100 kDa, with a more active fraction between 10 and 3 kDa [53]. The peptide profile as well as the antioxidant activity of the hydrolysates obtained after 3 h [53] and 2 h of reaction (this work) are similar. Therefore, similar values of total distribution of protein hydrolyzate and peptide fractions are also expected.

The three immobilized biocatalysts which presented the best results of operational stability (GLU-Chi pH7, GLU-Chi pH 10 and DVS-Chi pH 10) were evaluated as a biocatalyst in the tilapia skin gelatin hydrolysis aiming the production of bioactive peptides. The peptide profiles of the tilapia skin gelatin hydrolysate were analyzed by RP- HPLC (Fig. 7) and their antioxidant activity by DPPH free radical (Table 1).

Hydrolysates with higher antioxidant activity were obtained by the cleavage of tilapia skin gelatin using alcalase immobilized onto GLU-Chi as biocatalyst, at both pH values used for immobilization. The value achieved using these biocatalysts (35.5 and 36.7 μM Trolox Eq) was higher than that obtained with the free enzyme (29.4 μM Trolox Eq). Comparing the chromatographic profiles of the hydrolysates obtained using alcalase immobilized onto GLU-Chi (Fig. 7C and D), there is a certain similarity between them, with more intense peaks between 5 and 18 min, showing not only the wide diversity of molecules obtained through of the hydrolysis process, but also the polarity diversity, since between these times, the concentration of acetonitrile varied from 5 to 95 % (v/v). Compared to the profile obtained when free Alcalase (Fig. 7B) was the biocatalyst, highly hydrophilic peptides (present in the first retention time) were produced in less quantities by Chi-GLU. More hydrophobic peptides tend to have greater antioxidant activity [54], which may explain the difference in antioxidant activity between the hydrolyzates mentioned above. As for the hydrolyzate profile obtained using alcalase immobilized onto DVS-Chi pH 10 (Fig. 7E), it presents peaks closer to gelatin retention time, reflecting a less protein breakdown and, consecutively, less antioxidant activity (Table 1). The antioxidant activity is also related to the peptide molecular weights, as observed for the tilapia gelatin hydrolyzate obtained by free alcalase after 3 h of reaction [53] and other protein hydrolysates [54]. As mentioned before, activity and selectivity may depend on enzyme orientation onto the support [22,50], as well as the intensity of enzyme-support interactions [24]. Due to this, substrate specificity may have changed by different immobilization protocols, and thus the gelatin cleavage catalyzed by immobilized enzyme occurred at different protein regions generating several peptides, which may present distinct antioxidant activities [37].

The reusability of the immobilized enzymes was analyzed by carrying out three consecutive cycles of tilapia skin gelatin hydrolysis. Fig. 8 presents the relative antioxidant activity results of the formed peptides in each reaction cycle.

The biocatalyst obtained from the immobilization at pH 10 onto DVS-Chi did not present activity loss in those three cycles, performed at 55 °C, and, therefore, presented the best reusability performance for this reaction. On the other hand, differently of what happened in the operational stability assays during azocasein hydrolysis, the immobilized enzymes on GLU-Chi presented a considerable activity loss along with the studied cycles. The difference observed in that performance can be due to the stabilizing effect that proteins and peptides may present to the enzyme [8]. In the case of DVS-Chi biocatalyst, both substrate and hydrolysis products affect positively the stabilization toward temperature and other inactivation factors maintaining it totally activity during 3 cycles.

4. Conclusion

The different support activations and immobilization conditions offered varied features to the immobilization process and therefore to the obtained biocatalyst. Regarding the number of enzyme molecules fixed to the support, GLU-Chi has proven to be the most efficient support, achieving 100 % of enzyme immobilization in the first 15 h, at both studied pH, which was achieved by immobilization onto DVS-Chi in 72 h, only at pH 10. The biocatalysts produced presented great differences in the performance of the essayed hydrolysis, indicating the changing in the specificity of the substrate and enzyme stability generated by different immobilizations. In terms of activity, using NPA as a substrate, the biocatalysts obtained from immobilization onto GLU-Chi, at both pH, exhibited the best activity performances. However, in the azocasein hydrolysis, the GLU-Chi biocatalysts presented the lowest activity values and the GLY-Chi biocatalyst the best one, followed by the DVS-Chi biocatalysts. In the tilapia skin gelatin hydrolysis, the GLU-Chi biocatalyst presented the best performance. Regarding the enzyme stability, the GLU-Chi presented the best results in the thermal stability using NPA as a substrate and in the operational stability using azocasein. The DVS-Chi biocatalyst presented the best results of operational stability using the tilapia skin gelatin as a substrate. In summary, the different support and immobilization conditions essayed produced biocatalysts presenting different specificities and stabilities, which might be employed according to the operational needs.

All persons who meet authorship criteria are listed as authors, and all authors certify that they have participated sufficiently in the work to take public responsibility for the content, including participation in the concept, design, analysis, writing, or revision of the manuscript. Furthermore, each author certifies that this material or similar material has not been and will not be submitted to or published in any other publication before its appearance in the *Process Biochemistry*.

5. Authorship contributions

Author	Contribution
Kimberle Paiva dos Santos	Acquisition of data
	Analysis and/or interpretation of data
	Drafting the manuscript
	Approval of the version of the manuscript to be

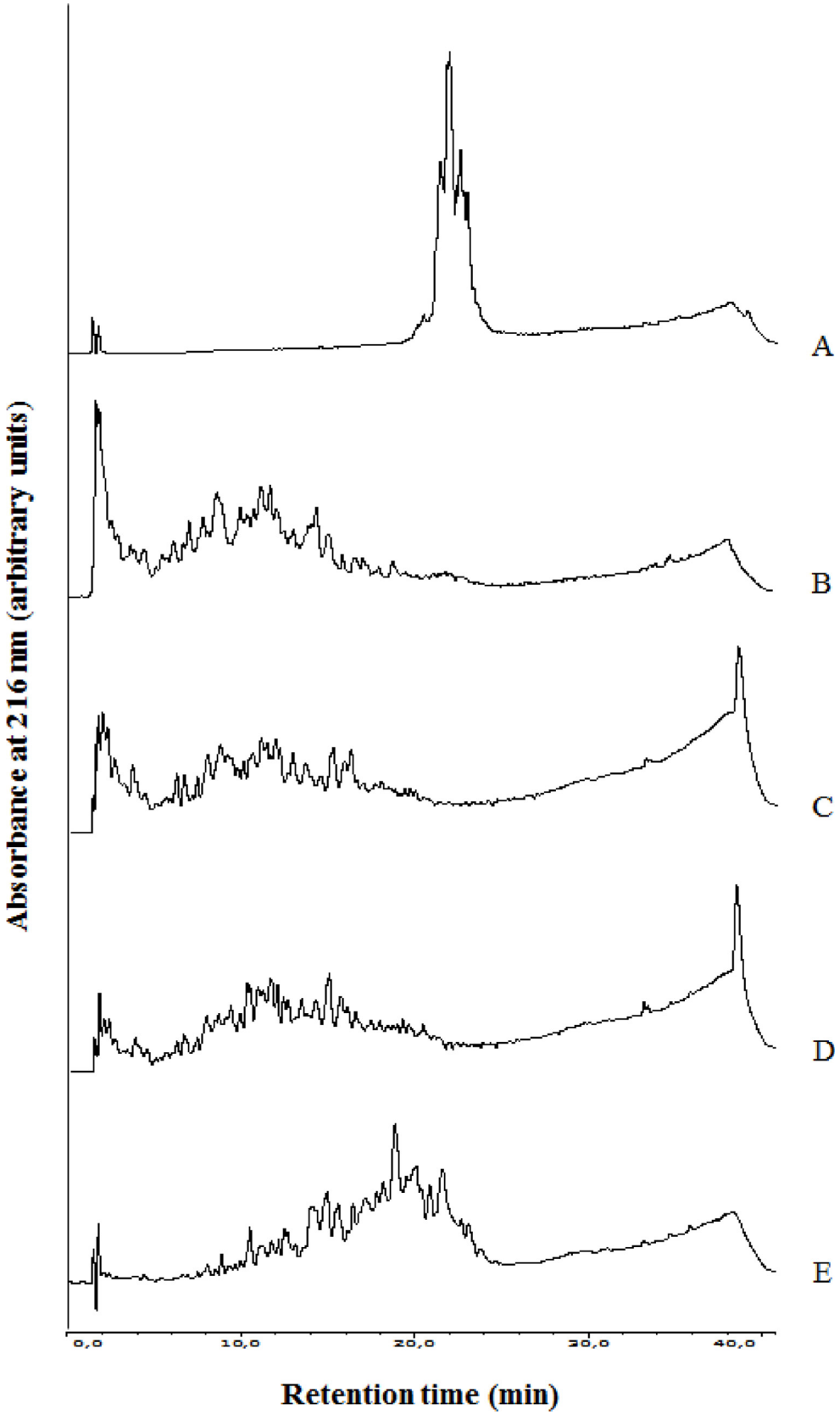

Fig. 7. Chromatographic peptide profiles of tilapia skin gelatin (A) hydrolysated with free alcalase (B), immobilized alcalase on GLU-Chi pH 7 (C), GLU-Chi pH 7 (D) or DVS-Chi pH 10 (E) in RP-HPLC.

Table 1
Antioxidant activity of the peptides obtained.

Biocatalyst	GLU-Chi pH7	GLU-Chi pH10	DVS-Chi pH10
Antioxidant activity (μM Trolox Eq)	35.5	36.7	22.0

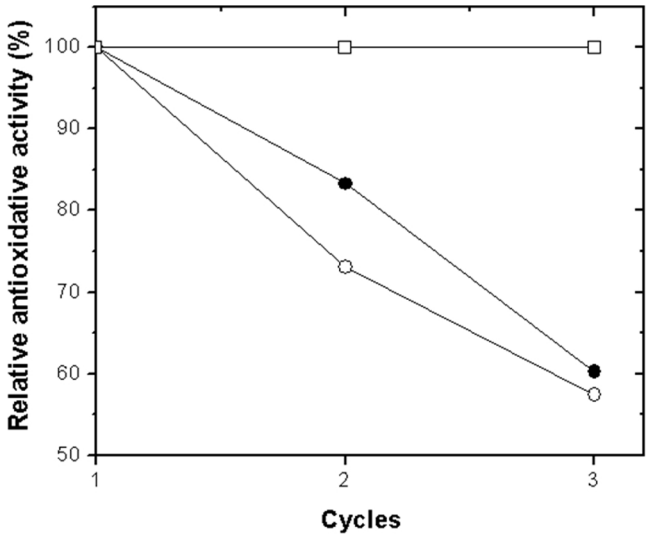

Fig. 8. Reusability performance in the bioactive peptide production reaction of the biocatalysts "GLU-Chi pH 7″ (●), "GLU-Chi pH 10″ (○) and "DVS-Chi pH 10″ (□).

Carolina Mellinger-SilvaAcquisition of data
Drafting the manuscript
Approval of the version of the manuscript to be
Ana Iraidy Santa BrígidaConception and design of study
Analysis and/or interpretation of data
Revising the manuscript critically for important intellectual content
Approval of the version of the manuscript to be
Luciana R. B. GonçalvesConception and design of study
Analysis and/or interpretation of data
Revising the manuscript critically for important intellectual content
Approval of the version of the manuscript to be

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The authors would like to thank the financial support and scholarships from the Brazilian agencies CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico), CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) and Funcap (Fundação Cearense de Apoio ao Desenvolvimento Científico e Tecnológico).

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.2020.06.019.

References

[1] L.Y. Chew, G.T. Toh, A. Ismail, Application of Proteases for the Production of Bioactive Peptides, Elsevier Inc., 2019, https://doi.org/10.1016/B978-0-12-813280-7.00015-3.
[2] M.G. Žuža, N.Z. Milašinović, M.M. Jonović, J.R. Jovanović, M.T. Kalagasidis Krušić, B.M. Bugarski, Z.D. Knežević-Jugović, Design and characterization of alcalase–chitosan conjugates as potential biocatalysts, Bioprocess Biosyst. Eng. 40 (2017) 1713–1723, https://doi.org/10.1007/s00449-017-1826-7.
[3] L. Ferreira, M.A. Ramos, J.S. Dordick, M.H. Gil, Influence of different silica derivatives in the immobilization and stabilization of a Bacillus licheniformis protease (Subtilisin Carlsberg), J. Mol. Catal., B Enzym. 21 (2003) 189–199, https://doi.org/10.1016/S1381-1177(02)00223-0.
[4] A. Osman, H.A. Goda, M. Abdel-hamid, S.M. Badran, J. Otte, LWT - Food Science and Technology Antibacterial peptides generated by Alcalase hydrolysis of goat whey, LWT - Food Sci. Technol. 65 (2016) 480–486, https://doi.org/10.1016/j.lwt.2015.08.043.
[5] G. Ballerini, M. Ortega, V. Giordanengo, Effects of enzymatic hydrolysis on the foaming properties of whey proteins, Int. Res. J. Agric. Food Sci. 1 (2016) 64–78.
[6] F. Zhong, X. Zhang, J. Ma, C.F. Shoemaker, Fractionation and identification of a novel hypocholesterolemic peptide derived from soy protein Alcalase hydrolysates, Food Res. Int. 40 (2007) 756–762, https://doi.org/10.1016/j.foodres.2007.01.005.
[7] M.M. Yust, J. Pedroche, J. Girón-Calle, M. Alaiz, F. Millán, J. Vioque, Production of ace inhibitory peptides by digestion of chickpea legumin with alcalase, Food Chem. 81 (2003) 363–369, https://doi.org/10.1016/S0308-8146(02)00431-4.
[8] P. Valencia, M. Pinto, S. Almonacid, Identification of the key mechanisms involved in the hydrolysis of fish protein by Alcalase, Process Biochem. 49 (2014) 258–264, https://doi.org/10.1016/j.procbio.2013.11.012.
[9] N. Charoenphun, B. Cheirsilp, Calcium-binding peptides derived from tilapia (Oreochromis niloticus) protein hydrolysate, Eur. Food Res. Technol. (2013) 57–63, https://doi.org/10.1007/s00217-012-1860-2.
[10] F. Guérard, L. Dufossé, D. De La Broise, A. Binet, Enzymatic hydrolysis of proteins from yellowfin tuna (Thunnus albacares) wastes using Alcalase, J. Mol. Catal., B Enzym. 11 (2001) 1051–1059, https://doi.org/10.1016/S1381-1177(00)00031-X.
[11] F. Hussain, S. Arana-Peña, R. Morellon-Sterling, O. Barbosa, S.A. Braham, S. Kamal, R. Fernandez-Lafuente, Further stabilization of alcalase immobilized on glyoxyl supports: amination plus modification with glutaraldehyde, Molecules 23 (2018), https://doi.org/10.3390/molecules23123188.
[12] C. Garcia-Galan, Á. Berenguer-Murcia, R. Fernandez-Lafuente, R.C. Rodrigues, Potential of different enzyme immobilization strategies to improve enzyme performance, Adv. Synth. Catal. 353 (2011) 2885–2904, https://doi.org/10.1002/adsc.201100534.
[13] D.N. Tran, J. Kenneth, J. Balkus, Perspective of recent progress in immobilization of enzymes, ACS Catal. 71 (2018) 41–57, https://doi.org/10.1163/1568525X-12342362.
[14] C.S. Bezerra, C.M. Gentil de Farias Lemos, M. de Sousa, L.R. Barros Goncalves, Enzyme immobilization onto renewable polymeric matrixes: past, present, and future trends, J. Appl. Polym. Sci. (2015), https://doi.org/10.1002/app.42125.
[15] J.C.S. Dos Santos, N. Rueda, R. Torres, O. Barbosa, L.R.B. Goncalves, R. Fernandez-Lafuente, Evaluation of divinylsulfone activated agarose to immobilize lipases and to tune their catalytic properties, Process Biochem. 50 (2015), https://doi.org/10.1016/j.procbio.2015.03.018.
[16] L.N. Corîci, A.E. Frissen, D.-J. van Zoelen, I.F. Eggen, F. Peter, C.M. Davidescu, C.G. Boeriu, Sol–gel immobilization of Alcalase from Bacillus licheniformis for application in the synthesis of C-terminal peptide amides, J. Mol. Catal., B Enzym. 73 (2011) 90–97, https://doi.org/10.1016/j.molcatb.2011.08.004.
[17] K. Hernandez, R. Fernandez-Lafuente, Control of protein immobilization: coupling immobilization and site-directed mutagenesis to improve biocatalyst or biosensor performance, Enzyme Microb. Technol. 48 (2011) 107–122, https://doi.org/10.1016/j.enzmictec.2010.10.003.
[18] U.M.F. de Oliveira, L.J.B.M. Matos, M.C. Souza, B.B. Pinheiro, J.C.S. Dos Santos, L.R.B. Gonçalves, Effect of the presence of surfactants and immobilization conditions on catalysts' properties of rhizomucor miehei lipase onto chitosan, Appl. Biochem. Biotechnol. 184 (2017) 1263–1285, https://doi.org/10.1007/s12010-017-2622-1.
[19] R. DiCosimo, J. McAuliffe, A.J. Poulose, G. Bohlmann, Industrial use of immobilized enzymes, Chem. Soc. Rev. 42 (2013) 6437–6474, https://doi.org/10.1039/c3cs35506c.
[20] P.G. Vazquez-Ortega, M.T. Alcaraz-Fructuoso, J.A. Rojas-Contreras, J. López-Miranda, R. Fernandez-Lafuente, Stabilization of dimeric β-glucosidase from Aspergillus niger via glutaraldehyde immobilization under different conditions, Enzyme Microb. Technol. 110 (2018) 38–45, https://doi.org/10.1016/j.enzmictec.2017.12.007.
[21] O. Barbosa, R. Torres, C. Ortiz, R. Fernandez-Lafuente, Versatility of glutaraldehyde to immobilize lipases: effect of the immobilization protocol on the properties of lipase B from Candida antarctica, Process Biochem. 47 (2012) 1220–1227, https://doi.org/10.1016/j.procbio.2012.04.019.
[22] N.S. Rios, M.P. Pinheiro, J.C.S. dos Santos, T. de, S. Fonseca, L.D. Lima, M.C. de Mattos, D.M.G. Freire, I.J. da Silva, E. Rodríguez-Aguado, L.R.B. Gonçalves, Strategies of covalent immobilization of a recombinant Candida antarctica lipase B on pore-expanded SBA-15 and its application in the kinetic resolution of (R, S)-Phenylethyl acetate, J. Mol. Catal., B Enzym. 133 (2016) 246–258, https://doi.org/10.1016/j.molcatb.2016.08.009.
[23] O. Barbosa, C. Ortiz, Á. Berenguer-Murcia, R. Torres, R.C. Rodrigues, R. Fernandez-Lafuente, Glutaraldehyde in bio-catalysts design: a useful crosslinker and a versatile tool in enzyme immobilization, RSC Adv. 4 (2014) 1583–1600, https://doi.org/10.1039/c3ra45991h.
[24] S. Ait Braham, F. Hussain, R. Morellon-Sterling, S. Kamal, J.F. Kornecki, O. Barbosa, D.E. Kati, R. Fernandez-Lafuente, Cooperativity of covalent attachment and ion

exchange on alcalase immobilization using glutaraldehyde chemistry: enzyme stabilization and improved proteolytic activity, Biotechnol. Prog. 35 (2019) e2768, https://doi.org/10.1002/btpr.2768.
[25] D.S. Rodrigues, A.A. Mendes, W.S. Adriano, L.R.B. Gonçalves, R.L.C. Giordano, Multipoint covalent immobilization of microbial lipase on chitosan and agarose activated by different methods, J. Mol. Catal., B Enzym. 51 (2008) 100–109, https://doi.org/10.1016/j.molcatb.2007.11.016.
[26] J.C.S. dos Santos, H.L. Bonazza, L.J.B.L. de Matos, E.A. Carneiro, O. Barbosa, R. Fernandez-Lafuente, L.R.B. Gonçalves, H.B. de Sant' Ana, R.S. Santiago-Aguiar, Immobilization of CALB on activated chitosan: application to enzymatic synthesis in supercritical and near-critical carbon dioxide, Biotechnol. Rep. Amst. (Amst) 14 (2017) 16–26, https://doi.org/10.1016/J.BTRE.2017.02.003.
[27] P.W. Tardioli, J. Pedroche, R.L.C. Giordano, R. Fernandez-Lafuente, J.M. Guisan, Hydrolysis of proteins by Immobilized-Stabilized alcalase-glyoxyl agarose, Biotechnol. Prog. 19 (2003) 352–360, https://doi.org/10.1021/bp025588n.
[28] B. Pessato, C. De Carvalho, O.L. Tavano, Whey protein isolate hydrolysates obtained with free and immobilized alcalase: characterization and detection of residual allergens, FRIN. (2016), https://doi.org/10.1016/j.foodres.2016.02.015.
[29] B.B. Pinheiro, N.S. Rios, E. Rodríguez Aguado, R. Fernandez-Lafuente, T.M. Freire, P.B.A. Fechine, J.C.S. dos Santos, L.R.B. Gonçalves, Chitosan activated with divinyl sulfone: a new heterofunctional support for enzyme immobilization. Application in the immobilization of lipase B from Candida antarctica, Int. J. Biol. Macromol. 130 (2019) 798–809, https://doi.org/10.1016/j.ijbiomac.2019.02.145.
[30] J.C.S. dos Santos, N. Rueda, O. Barbosa, J.F. Fernández-Sánchez, A.L. Medina-Castillo, T. Ramón-Márquez, M.C. Arias-Martos, M.C. Millán-Linares, J. Pedroche, M.D.M. Yust, L.R.B. Gonçalves, R. Fernandez-Lafuente, Characterization of supports activated with divinyl sulfone as a tool to immobilize and stabilize enzymes via multipoint covalent attachment. Application to chymotrypsin, RSC Adv. 5 (2015) 20639–20649, https://doi.org/10.1039/C4RA16926C.
[31] J.C.S. Santos, N. Rueda, O. Barbosa, M.D.C. Millán-Linares, J. Pedroche, M. del Mar Yuste, L.R.B. Gonçalves, R. Fernandez-Lafuente, Bovine trypsin immobilization on agarose activated with divinylsulfone: improved activity and stability via multipoint covalent attachment, J. Mol. Catal., B Enzym. 117 (2015) 38–44, https://doi.org/10.1016/j.molcatb.2015.04.008.
[32] L. Dal Magro, J.F. Kornecki, M.P. Klein, R.C. Rodrigues, R. Fernandez-Lafuente, Optimized immobilization of polygalacturonase from Aspergillus niger following different protocols: improved stability and activity under drastic conditions, Int. J. Biol. Macromol. 138 (2019) 234–243, https://doi.org/10.1016/j.ijbiomac.2019.07.092.
[33] W.S. Adriano, V. Veredas, C.C. Santana, L.R.B. Goncalves, Adsorption of amoxicillin on chitosan beads: kinetics, equilibrium and validation of finite bath models, Biochem. Eng. J. (2005), https://doi.org/10.1016/j.bej.2005.08.010.
[34] J. Guisán, Aldehyde-agarose gels as activated supports for immobilization-stabilization of enzymes, Enzyme Microb. Technol. 10 (1988) 375–382, https://doi.org/10.1016/0141-0229(88)90018-X.
[35] J. Charney, R.M. Tomarelli, A colorimetric method for the determination of the proteolytic activity of duodenal juice, J. Biol. Chem. 171 (1947) 501–505 http://www.ncbi.nlm.nih.gov/pubmed/20272088.
[36] J.P. Henley, A. Sadana, Categorization of enzyme deactivations using a series-type mechanism, Enzyme Microb. Technol. 7 (1985) 50–60, https://doi.org/10.1016/0141-0229(85)90013-4.
[37] S. Choonpicharn, S. Jaturasitha, N. Rakariyatham, N. Suree, H. Niamsup, Antioxidant and antihypertensive activity of gelatin hydrolysate from Nile tilapia skin, J. Food Sci. Technol. 52 (2015) 3134–3139, https://doi.org/10.1007/s13197-014-1581-6.
[38] J. Yang, W. Liang, C. Chow, K.J. Siebert, Process for the production of tilapia retorted skin gelatin hydrolysates with optimized antioxidative properties, Process. Biochem. 44 (2009) 1152–1157, https://doi.org/10.1016/j.procbio.2009.06.013.
[39] J.A. Silva, G.P. Macedo, D.S. Rodrigues, R.L.C. Giordano, L.R.B. Gonçalves, Immobilization of Candida antarctica lipase B by covalent attachment on chitosan-based hydrogels using different support activation strategies, Biochem. Eng. J. 60 (2012) 16–24, https://doi.org/10.1016/j.bej.2011.09.011.
[40] L. Betancor, F. López-Gallego, A. Hidalgo, N. Alonso-Morales, G. Dellamora-Ortiz, C. Mateo, R. Fernández-Lafuente, J.M. Guisán, Different mechanisms of protein immobilization on glutaraldehyde activated supports: effect of support activation and immobilization conditions, Enzyme Microb. Technol. 39 (2006) 877–882, https://doi.org/10.1016/j.enzmictec.2006.01.014.
[41] L.M. Pinotti, P.W. Tardioli, C.S. Farinas, G. Fernández-lorente, A.H. Orrego, J.M. Guisan, Stabilization of Glycosylated β -Glucosidase by Intramolecular Crosslinking Between Oxidized Glycosidic Chains and Lysine Residues, Appl. Biochem. Biotechnol. (2020).
[42] C. Mateo, J.M. Palomo, G. Fernandez-Lorente, J.M. Guisan, R. Fernandez-Lafuente, Improvement of enzyme activity, stability and selectivity via immobilization techniques, Enzyme Microb. Technol. 40 (2007) 1451–1463, https://doi.org/10.1016/j.enzmictec.2007.01.018.
[43] O. Barbosa, R. Torres, C. Ortiz, A. Berenguer-Murcia, R.C. Rodrigues, R. Fernandez-Lafuente, Heterofunctional supports in enzyme immobilization: from traditional immobilization protocols to opportunities in tuning enzyme properties, Biomacromolecules. 14 (2013) 2433–2462, https://doi.org/10.1021/bm400762h.
[44] V.M. Balcão, M.M.D.C. Vila, Structural and functional stabilization of protein entities: state-of-the-art, Adv. Drug Deliv. Rev. 93 (2015) 25–41, https://doi.org/10.1016/j.addr.2014.10.005.
[45] M. Bilal, M. Asgher, H. Cheng, Y. Yan, H.M.N.N. Iqbal, Multi-point enzyme immobilization, surface chemistry, and novel platforms: a paradigm shift in biocatalyst design, Crit. Rev. Biotechnol. 39 (2019) 202–219, https://doi.org/10.1080/07388551.2018.1531822.
[46] P. Torres, F. Batista-Viera, Production of D-tagatose and D-fructose from whey by co-immobilized enzymatic system, Mol. Catal. (2019).
[47] E.-H. Siar, H. Zaak, J.F. Kornecki, M.N. Zidoune, O. Barbosa, R. Fernandez-Lafuente, Stabilization of ficin extract by immobilization on glyoxyl agarose. Preliminary characterization of the biocatalyst performance in hydrolysis of proteins, Process Biochem. 58 (2017) 98–104, https://doi.org/10.1016/j.procbio.2017.04.009.
[48] I. Migneault, C. Dartiguenave, M.J. Bertrand, K.C. Waldron, Glutaraldehyde: Behavior in aqueous solution, reaction with proteins, and application to enzyme crosslinking, Biotechniques. 37 (2004) 790–802.
[49] P. Zucca, R. Fernandez-Lafuente, E. Sanjust, Agarose and its derivatives as supports for enzyme immobilization, Molecules 21 (2016) 1–25, https://doi.org/10.3390/molecules21111577.
[50] C.R. Matte, R. Bussamara, J. Dupont, R.C. Rodrigues, P.F. Hertz, M.A.Z. Ayub, Immobilization of Thermomyces lanuginosus lipase by different techniques on Immobead 150 support: characterization and applications, Appl. Biochem. Biotechnol. 172 (2014) 2507–2520, https://doi.org/10.1007/s12010-013-0702-4.
[51] O.L. Tavano, A. Berenguer-Murcia, F. Secundo, R. Fernandez-Lafuente, Biotechnological applications of proteases in food technology, Compr. Rev. Food Sci. Food Saf. 17 (2018) 412–436, https://doi.org/10.1111/1541-4337.12326.
[52] J. Pedroche, M. del Mar Yust, C. Mateo, R. Fernández-Lafuente, J. Girón-Calle, M. Alaiz, J. Vioque, J.M. Guisán, F. Millán, Effect of the support and experimental conditions in the intensity of the multipoint covalent attachment of proteins on glyoxyl-agarose supports: correlation between enzyme–support linkages and thermal stability, Enzyme Microb. Technol. 40 (2007) 1160–1166, https://doi.org/10.1016/j.enzmictec.2006.08.023.
[53] H.L.S. Lima, C. Gonçalves, M.Â. Cerqueira, E.S. do Nascimento, M.F. Gama, M.F. Rosa, M. de F. Borges, L.M. Pastrana, A.I.S. Brígida, Bacterial cellulose nanofiber-based films incorporating gelatin hydrolysate from tilapia skin: production, characterization and cytotoxicity assessment, Cellulose. 25 (2018) 6011–6029, https://doi.org/10.1007/s10570-018-1983-0.
[54] T. Bin Zou, T.P. He, H. Bin Li, H.W. Tang, E.Q. Xia, The structure-activity relationship of the antioxidant peptides from natural proteins, Molecules. 21 (2016) 1–14, https://doi.org/10.3390/molecules21010072.