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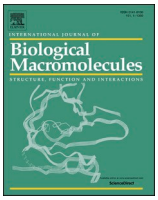

Optimizing the activation of agarose beads with divinyl sulfone for enzyme immobilization and stabilization

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ABSTRACT

The focus of the present work is to find the optimal conditions for the activation of agarose beads with divinyl sulfone (DVS). The reactivity of the vinyl sulfone groups in the support was checked by the support capacity to react with ethylamine; via elemental analysis. In addition, trypsin was used as a model enzyme to test the immobilization and stabilization capabilities of the different supports. The higher the pH, the more vinyl sulfone groups are incorporated into the support, but lower reactivity versus ethylamine is observed. Too long activation times led to similar results. A N/S ratio of 1 means that all vinyl sulfone groups were reactive, and it was always lower than tis figure. The N in the support was 50 % of the amount observed for glyoxyl supports activated with ethylenediamine, suggesting the VS polymerization may be a likely explanation for this result. The higher N/S ratio in the support (modified with ethylamine), the higher the obtained stabilization, very likely by the lower polymerization of the vinyl sulfone on the support. We propose 360 mM divinyl sulfone, at pH 11.5 and 2 h as optimal conditions to reach the highest enzyme stabilization by immobilization in this support.

1. Introduction

With the rise of enzyme biocatalysis [1–5], the development of strategies to further improve enzyme features is under strong development. That way, metagenomics [6–9], directed evolution [10–12], rational site-directed mutagenesis [13–15], protein chemical modification [16–19] and enzyme immobilization [20–23] are rapidly evolving to give solutions to the effective design of industrial enzyme biocatalysts. Enzyme immobilization was initially developed to solve the problem of enzyme recovery and reuse, as initially enzymes were very expensive biocatalysts [23,24]. Moreover, these heterogeneous biocatalysts permitted us to take advantage of the heterogeneous catalysis and simplify the product downstream [22,23,25,26]. Soon, some researchers tried to couple this "compulsory" step in the biocatalyst development as a solution to other enzyme limitations. Nowadays, it has been shown that immobilization can improve many enzyme features, such as enzyme activity, selectivity, specificity, inhibitions, [20,21,27] even enzyme purification may be coupled to a proper enzyme immobilization protocol [28–30]. However, the initial target of enzyme immobilization was the stabilization via immobilization of the enzyme. Immobilization inside a porous support prevented some inactivation causes of the enzyme, such as interactions with gas bubbles or drops of solvents, enzyme aggregation or proteolysis [31]. The Russian school showed how multipoint covalent immobilization can actually increase enzyme rigidity, and this produces positive effects on enzyme stability [32–38]. Moreover, multi-subunit immobilization of multimeric enzymes [39–42], fixation of more stable structures [43,44], generation of favorable nano-environments [45–50], etc. could also promote enzyme stabilization. Nevertheless, to reach these objectives, the immobilization protocol should be properly designed, otherwise the immobilized enzyme stability can be even lower than that of the free enzyme [51,52].

In this context, glyoxyl, glutaraldehyde or epoxy supports may be

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highlighted as very suitable ones to enable the enzyme stabilization via multipoint covalent immobilization. However, they have some limitations. Glyoxyl-supports may be the most efficient matrices to get multipoint covalent immobilization [53], as they direct the immobilization by the area of the enzyme that is the richest one in reactive groups (facilitating the enzyme-support multipoint reaction) [54], are very stable, with short spacer arms and scarce steric hindrances towards the reaction with the reactive groups of the enzyme. However, they must be used at alkaline pH values to produce these results, and require a final reduction with sodium borohydride, conditions that may not be compatible with the stability of some enzymes. Moreover they only react with primary amino groups, and enzymes with low amounts of Lys cannot even become immobilized, as immobilization is only produced via several enzyme-support reactions [53]. The use of glutaraldehyde to immobilize enzyme is another way to give high enzyme stabilizations. Albeit being very popular, the exact form of glutaraldehyde that permits enzyme immobilization is not known, and it belongs to a family that may suffer many modifications (e.g., it is photosensible) [55–58]. Aminated supports modified with glutaraldehyde permit only a moderate multipoint covalent immobilization, as the glutaraldehyde groups are not very stable at alkaline pH value and only non-ionized amino groups are reactive [55]. The highest stabilizations are generally obtained treating the previously ionically exchanged enzyme on aminated supports with glutaraldehyde [59]. This glutaraldehyde modification can lead to enzyme inactivation in some instances. As in the case of glyoxyl, the main group in the enzyme that is used in the enzyme-support reaction is the primary amnion group. As an advantage, it is not necessary to use a reaction end-point, as the reactivity is lost after some time. This support can be utilized to immobilize several enzymes with different stabilities enabling the reuse of the most stable one [60,61]. Supports with epoxide groups seem to solve some of the problems: epoxides can react with amino groups, thiol, imidazole, phenol and even carboxylic groups, extending the number of groups that can participate in the enzyme-support reaction, and that way, the possibilities of getting high multipoint covalent immobilization [62]. The spacer arm may be short and the steric hindrances for the enzyme-support reaction are low, being stable even at alkaline pH value [63]. The supports are supplied as ready to use ones, and have provided some successes in enzyme stabilization [64–67]. However, the reactivity is low, and enzyme immobilization must proceed via a previous enzyme incorporation to the support by physical or chemical methods, although this permits to generate heterofunctional supports increasing the versatility of the protocol [68], the obtained results tend to be poorer than using the other immobilization methods. As reaction end point, the support blocking with nucleophiles may be utilized. In this context, the use of supports activated with divinyl sulfone, reported a long time ago as a suitable support-activating reagent for enzyme immobilization [69–80], has been recently reported to fulfil many of the requirements to become very suitable to produce very intense enzyme-support multipoint covalent attachment, and that way to give reach to very high stabilization of many enzymes [81,82]. As epoxides, they can react with different groups of proteins (thiol, imidazole, amino, phenol) (Fig. 1), they are quite stable in the 5–10 pH range, there are low steric hindrances to the reaction of the vinyl group with the groups of the enzyme [81,82], etc. Its main problem is that the spacer arm is relatively long: rigidification of the enzyme derived from fixing the relative positions of the involved group, which can only move the length of the spacer arm [81,82]. However, this can also permit the establishment of more enzyme-support bonds, and the final balance will depend on each specific enzyme and inactivation conditions. The end point of the enzyme-support reaction may be achieved by blocking the remaining vinyl sulfone groups in the support with different reagents. This has shown to be a last opportunity to tune the immobilized enzyme features, as the enzyme support interactions between the blocked support (that also alters the enzyme nanoenvironment) may alter the enzyme structure, and that way, its activity and stability, in both, monofunctional vinyl sulfone supports or heterofunctional supports

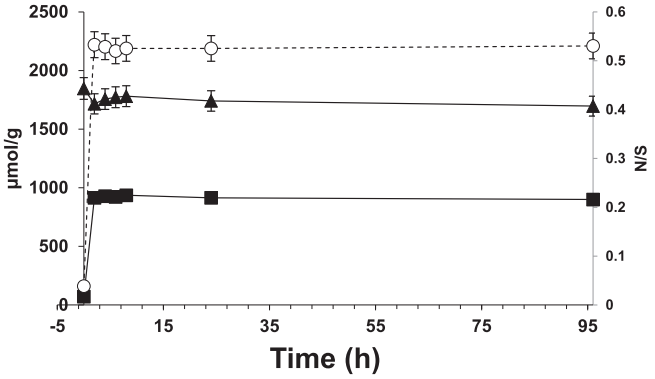

Fig. 1. Scheme of the immobilization of enzymes in vinyl sulfone agarose.

containing vinyl sulfone groups [71,76,79,83–85]. Moreover, it allows the coimmobilization of enzymes, first via covalent immobilization and then, via physical adsorption after biocatalyst blocking. This permits to design strategies to reuse the most stable enzyme(s) (that should be the enzyme(s) covalently immobilized) after the inactivation of the less stable enzymes(s) [80,86–88]. Not only enzyme stabilization may be achieved using these supports, but also this methodology may be extended to activate supports bearing hydroxyl, thiol, amino (primary or secondary), imidazole, etc., becoming a quite general support activation group.

In this paper, we have focused on the optimization of the activation of agarose beads with divinyl sulfone. Agarose beads have the advantage of being a fully inert support, the only capacity to interact with proteins being the introduced groups [89]. The activation of the supports is mainly via the primary hydroxy group of the polymer, during the activation it is possible that some vinyl sulfone groups can open by reacting with water (mainly at alkaline pH value), or that some polymerization with free divinyl sulfone can occur. The target of the support activation with divinyl sulfone should be to get the maximum amount of reactive vinyl sulfone groups on the support. To determine this, the activated supports were incubated in ethylamine, and the amount of S and N of the different supports was determined by elemental analysis. The main objective of this paper is, that way, to establish the optimal conditions for the activation of this support with the aim of reaching the highest stabilization of the enzyme, together with analyzing the effects on immobilization rate and expressed activity. As model enzyme to check the effect of the activation protocol on the final immobilized enzyme features, we have employed bovine trypsin an interesting protease that has been successfully immobilized/stabilized on this support [82].

2. Materials and methods

2.1. Materials

α-chymotrypsin from bovine pancreas (≥ 40 units/mg of protein), N-benzoyl-L-tyrosine p-nitro-anilide (BTPNA), glycine and ethylenamine were purchased form Sigma-Aldrich (Darmstadt, Germany). 4 % BCL agarose beads (crosslinked agarose beads) standard (50–150 µm) were obtained from Agarose Bead Technologies (Burgos, Spain). Divinyl sulfone (stabilized with hydroquinone) was purchased from Tokyo Chemical Industry Europe (Zwijndrecht, Belgium.). All the other reagents used were of analytical grade.

2.2. Methods

All experiments have been performed by triplicate and the results are presented as the mean value of at least 3 measurements, with their standard deviation.

2.2.1. Preparation of vinyl sulfone-agarose supports

A 200 mL solution of 333 mM sodium carbonate was prepared at different pH values (10.5–12.5), then a mass of 10 g of 4 % BCL agarose beads was suspended, and finally 7.5 mL of divinyl sulfone was added under continues stirring. The mixture was left under gentle agitation for the indicated times. Then, the support was vacuum filtered, thoroughly washed with distilled water and preserved at 4 °C until utilization.

2.2.2. Modification of the vinyl sulfone agarose supports with ethylamine

The amount of reactive vinyl sulfone groups was determined by modification with ethyl amine. This was performed by incubations 1 g of the studied vinyl sulfone support in 2 M ethyl amine at pH 10.5 for 16 h at 25°. Later, the support was washed with an excess of distilled water, followed by a washing with acetone and dried by storage at 50 °C for 24 h. Then, the support was submitted to elemental analysis.

2.2.3. Elemental analysis of the supports

Inert, vinyl sulfone and ethylamine-vinyl sulfone agarose beads, all dried as described in section 2.2.2, were submitted to elemental analysis. COHN elemental analyses were carried out employing a Leco 932 CHNS combustion microanalyzer (accredited range: % C: 0.4–82.42; % H: 2.75–6.71; % N: 0.45–20.13; % S: 0.57–26.699). The Elemental Microanalysis Unit of the Chemical Technologies inside the Research Assistance Centre (Centros de Asistencia a la Investigación, CAI) of the Complutense University of Madrid made the analyses.

2.2.4. Determination of the activity of trypsin

To quantify the activity of free and immobilized trypsin the increase of absorbance caused by the release of *p*-nitroaniline in the reaction medium was measured at 405 nm ($\varepsilon = 9960\ M^{-1}\ cm^{-1}$ under the described conditions [90]) when hydrolysing BAPNA. The final concentration of this substrate was 2 mM and it was prepared in a pH 7 at 25 °C solution that contained 70 % 50 mM of a sodium phosphate / 30 % of ethanol [91].

The reaction was carried out by adding a desired volume of enzymatic solution or solution to a cuvette containing 2.5 mL of the desired substrate solution, placed in a thermo-regulated spectrophotometer with magnetic agitation.

2.2.5. Trypsin immobilization in divinyl sulfone-agarose beads

The immobilization of trypsin in agarose beads activated with divinyl sulfone was performed in 50 mM of a sodium carbonate at pH 10.05 and 25 °C for 6 h. A ratio of 1 g of support for 5 mL of a 10 mg /mL trypsin solution containing 3 mM of benzamidine [92]. This immobilization suspension was kept under gentle stirring until entire enzyme activity was immobilized. To monitor the immobilization, different samples of the suspension and supernatant were obtained at certain times and their activity measured as described in the above section, using a solution prepared under identical conditions where inert agarose was used as reference (no immobilization was observed in this inert support). After this first step, the biocatalysts were vacuum filtered, washed with distilled water and weighted, then they were resuspended them in a 2 M glycine solution (1 g biocatalyst/10 mL solution) to block the vinyl sulfone groups in the support that had not reacted with the proteins. Finally, the biocatalysts were once more vacuum filtered, washed thoroughly with distilled water and stored at 4 °C.

2.2.6. Thermal inactivation of the biocatalysts

Thermal inactivations of the different biocatalysts produced were carried out in heated water baths. The different biocatalysts were resuspended in 25 mM of either sodium carbonate pH 9, sodium phosphate pH 7 or sodium acetate pH 5, using a ratio of 10 mL for each g of biocatalyst. The temperature was selected to provide reliable inactivation courses, in this case 72 °C was the selected one at pH 5 and 7 and 60 °C at pH 9. The residual activity was measured as described in the enzymatic assay section at several times throughout the inactivation process.

3. Results and discussion

3.1. Modification of the vinyl sulfone supports with ethylamine

First, we evaluated the conditions where the vinyl sulfone groups modification with ethyl amine reached a maximum value, that should reflect the amount of active vinyl sulfone (Fig. 2). This means that we should ensure that we really determined the amount of active vinyl sulfone groups in the support by fully reacting them with ethylamine. This is possible because agarose beads almost lack of N and S in its composition. That way, each mol of vinyl sulfone that reacts with the support provide a mol of S, and each mol of ethylamine that reacts with the support provide a mol of N. To this preliminary goal, we selected a support activated for 2 h at pH 11 using 360 mM of divinyl sulfone. Fig. 2 shows that in only 2 h, we reached almost the maximum modification of the vinyl sulfone support with ethylamine under the modification conditions (and that showed that 52–53 % of the vinyl sulfone groups were reactive and modified with ethylamine, that is almost 50 % of the vinyl groups in the support were not reactive). The N/S value remained constant over time. We selected 16 h of incubation to perform further studies, as 2 h of activation time may be complicated if extrapolated at high volumes of support (e.g., in industrial processes).

3.2. Effect of reaction time, pH and divinyl sulfone concentration in the activation of agarose beads with vinyl sulfone

Next, we evaluated the effect of the activation pH value on the modification of agarose beads with divinyl sulfone. This parameter determines the reactivity of the primary hydroxy group in the agarose structure. However, it can also condition the stability of the divinyl sulfone and the introduced vinyl sulfone groups. Fig. 3 shows the results. At pHs 10 and 10.5, using 360 mM of vinyl sulfone and a reaction time of 0.5 h, the amount of S (and consequently of vinyl sulfone groups) is very small, although they almost give one mol of N per mol of S after modification with ethylamine, showing that most of the introduced groups remained active. At pH 11 the amount of vinyl sulfone in the support increased, maintaining a good N/S ratio after ethylamine modification. The amount of vinyl sulfone continued increasing at pH 11, 12 and 12.5, but after modification with ethylamine, the N/S ratio is still smaller. Higher pH values promoted a rapid formation of a precipitate, which was attributed to the polymerization of the divinyl sulfone. That way, at this short reaction time, alkaline pH values favoured the modification with divinyl sulfone and, although with a percentage of vinyl sulfone which was rendered non-reactive, alkaline pH values gave the higher amount of reactive vinyl sulfone.

Next, we studied the effect of time at pH 10.5, 11.5 and 12.5 (Fig. 4). At pH 10.5 and 11.5, it is evident that the longer the time, the higher amount of S could be incorporated to the support. At pH 10, the reactivity versus ethylamine of the activated support decrease when prolonging time. In fact, at 6 h it reached a maximum capacity to react with ethylamine (already no permitting to have a ratio N/S of 1), decreasing after 24 h. That way, even at pH 10.5 it looks that it may be difficult to have a high amount of vinyl sulfone groups introduced in the support mantling full reactivity (Fig. 4). At pH 11.5, times longer than 6 h produced the formation of a solid in the reference (a solution under the same conditions in absence of support). That way, the reaction was studied only until 6 h. Although between 2 and 6 h a significant amount of new vinyl sulfone groups are incorporated (reflected by the S in the support), the maximum reactivity is reached after only 2 h and then remains almost constant over time (reflected by the N incorporated after reaction with ethylamine) (Fig. 4). At pH 12.5, reaction time longer than 1 h produced a solid in divinyl sulfone solution, that way, the maximum time of activation was fixed at 1 h. The Fig. 4 shows that the amount of S in the support is lower after 15 min, reached a maximum after 30 min

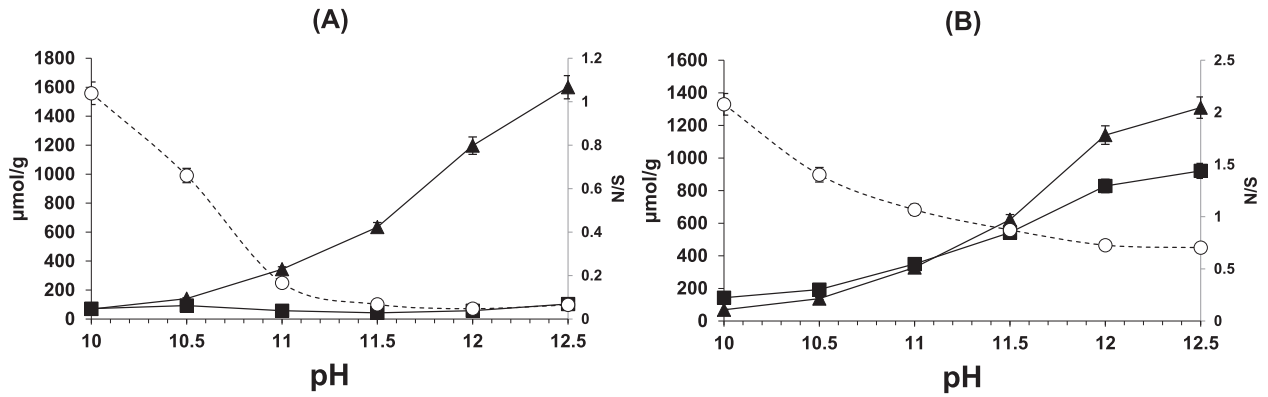

Fig. 2. Elemental analysis of a vinyl sulfone support modified with ethylamine at different times. Solid squares: nitrogen µmol/g; Solid triangles: sulfur µmol/g; empty circles: nitrogen/sulfur ratio.

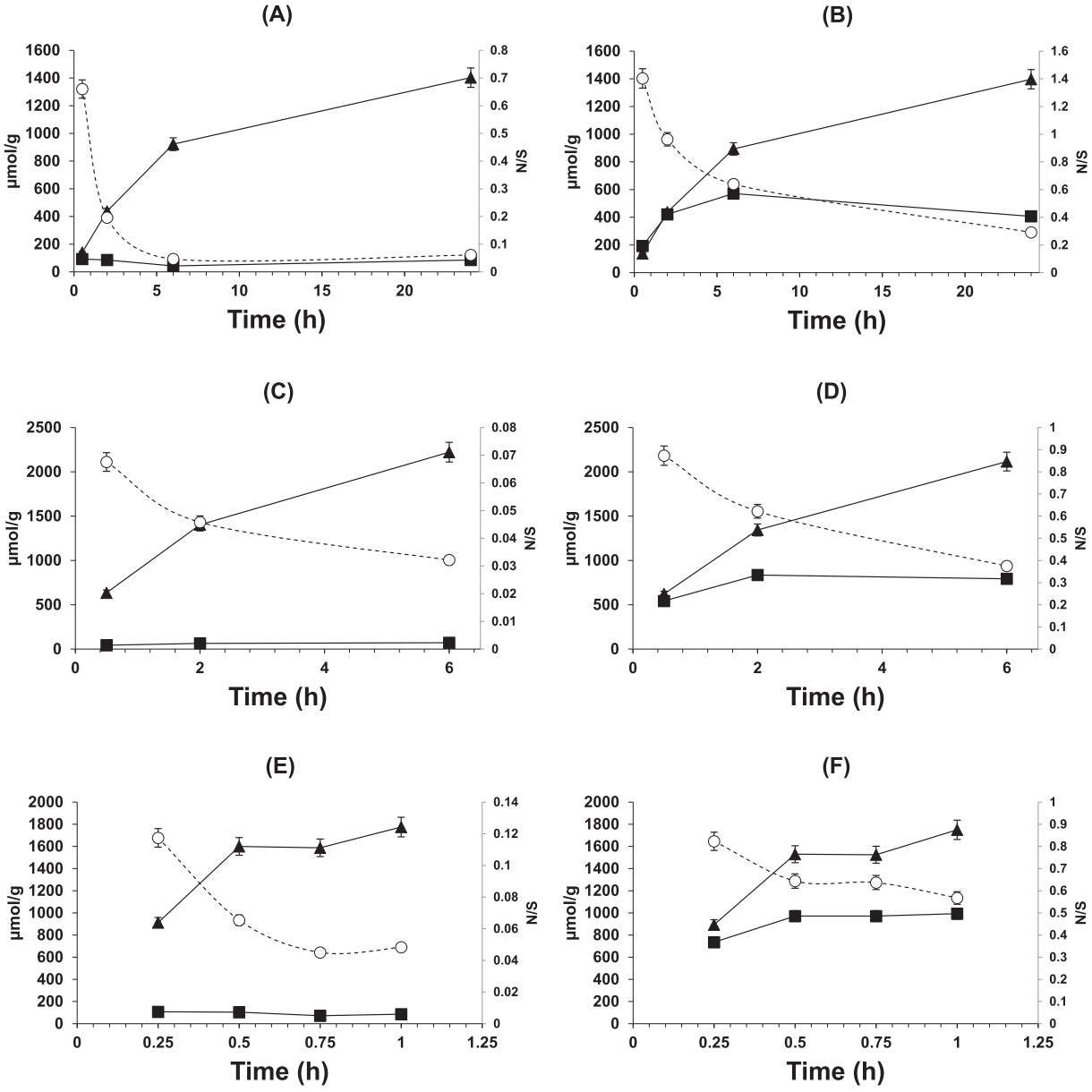

Fig. 3. Elemental analysis of different agarose supports modified with vinyl sulfone for 0.5 h at different pHs. (A) vinyl sulfone agarose and (B) vinyl sulfone agarose blocked with ethylamine. Solid squares: nitrogen µmol/g; Solid triangles: sulfur µmol/g; empty circles: nitrogen/sulfur ratio.

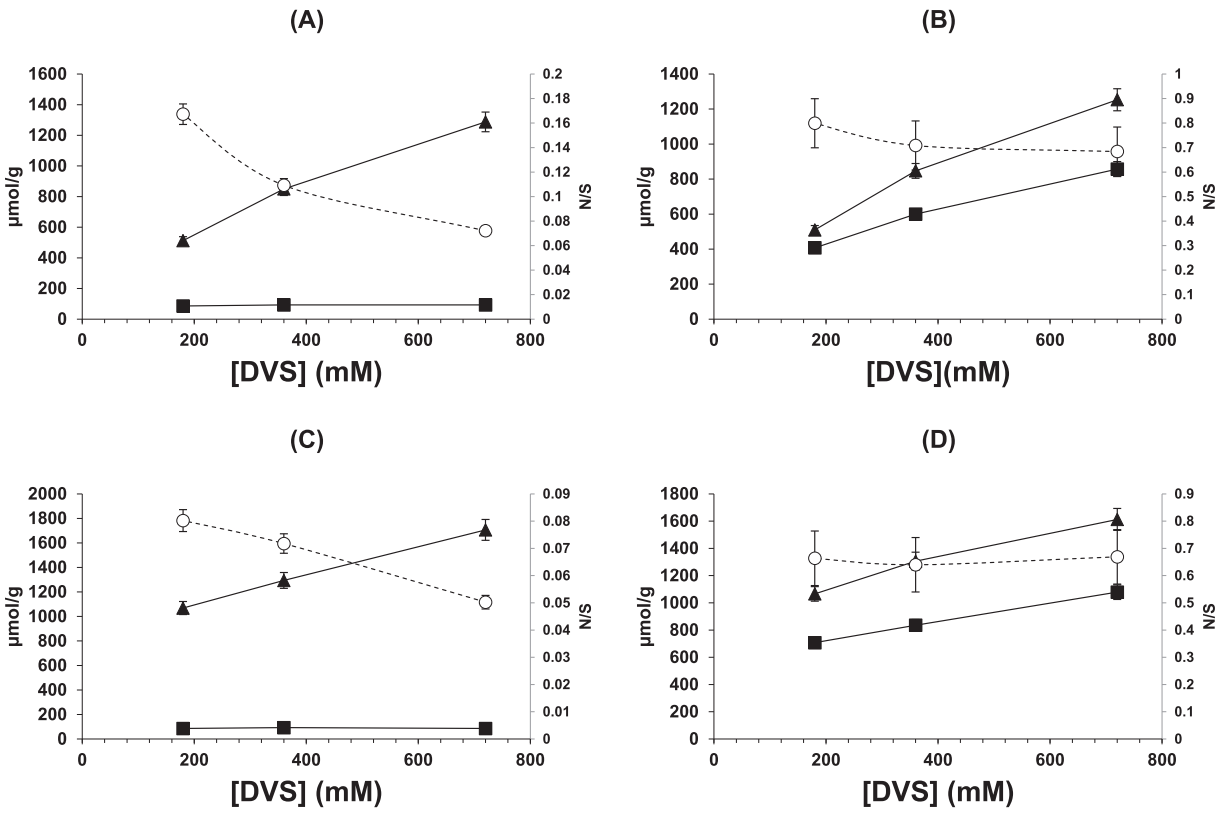

Fig. 4. Elemental analysis of different agarose supports modified with vinyl sulfone at different pHs and different modification times. (A) pH 10.5, (B) pH 10.5 blocked with ethylamine, (C) pH 11.5, (D) pH 11.5 blocked with ethylamine, (E) pH 12.5, (F) pH 12.5 blocked with ethylamine. Solid squares: nitrogen µmol/g; Solid triangles: sulfur µmol/g; empty circles: nitrogen/sulfur ratio.

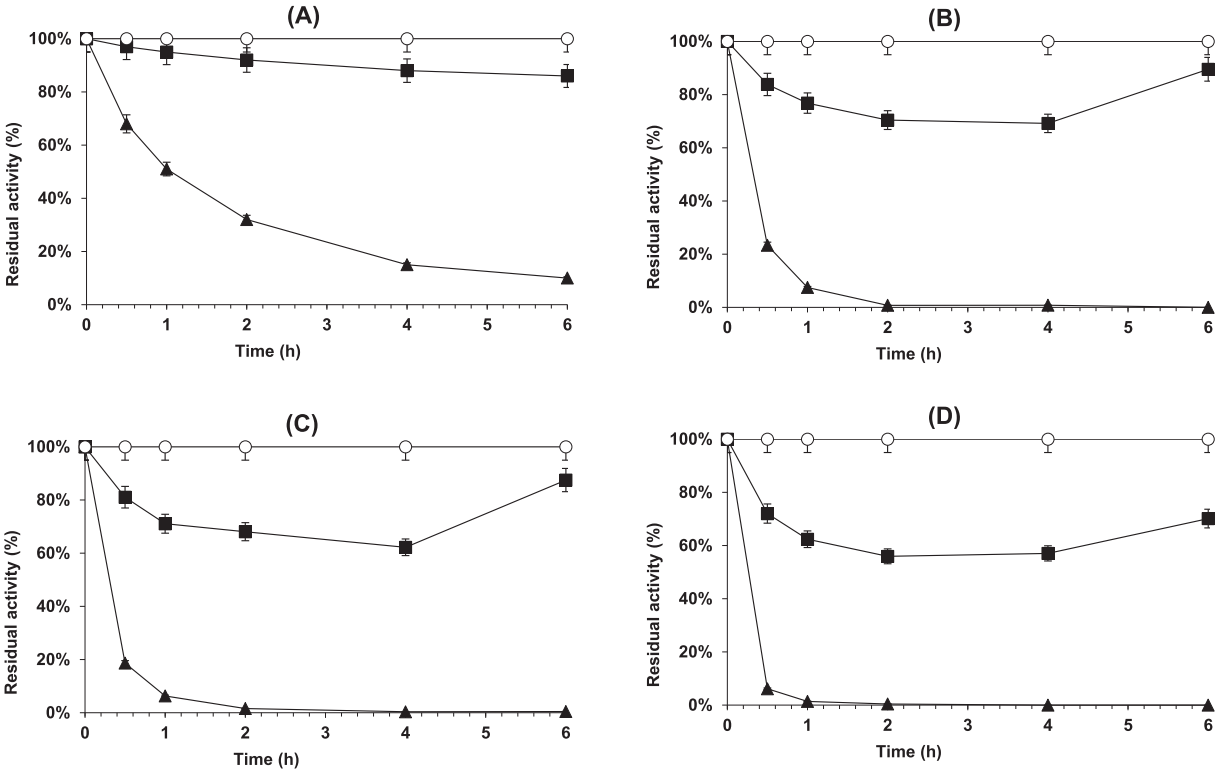

Fig. 5. Elemental analysis of different agarose supports modified with vinyl sulfone at several concentrations and different pHs. (A) pH 10.5, (B) pH 10.5 blocked with ethylamine, (C) pH 11.5 and (D) pH 11.5 blocked with ethylamine. Solid squares: nitrogen µmol/g; Solid triangles: sulfur µmol/g; empty circles: nitrogen/sulfur ratio.

and remained almost unaltered when prolonging the incubation time to 45 or 60 min. Curiously, a similar situation is observed in the capacity to react with ethylamine, remains almost unaltered after 1 h of activation, suggesting that the vinyl sulfone groups were not inactivated under these conditions (Fig. 4).

Finally we analysed the effect of the divinyl sulfone concentration at several pH values and incubation times (Fig. 5). At pH 12.5 it was no possible to increase the concentration, as a fast polymerization occurred. The changes in concentration of vinyl sulfone did not permit to get better modification of the support, just slowdown or accelerate the reaction, making the use of higher concentrations risky by making the polymerization of divinyl sulfone in the support easier.

3.3. Effect of the support activation protocol in the support performance to immobilize trypsin

Fig. 6 shows the immobilization courses of trypsin in 4 different supports, with different amount of S and N/S ratios after ethylamine modification (Fig. 4). The faster immobilizations were observed on supports activated at pH 12.5 for 30 min, followed by the supports prepared at pH 11.5 at 3 and 6 h (differences between the immobilization in both supports were short), and the trypsin immobilization was the slowest one using the support activated at pH 10.5 for 24 h. These results fit with the amount of N that the supports were able to incorporate after ethylamine modification (Fig. 4), that indicate the amount of reactive group in the supports. Recovered activity was slightly higher using the support activated at pH 10, followed by the enzyme immobilized in the supports activated at pH 11.5 and being the last the recovered activity using the support activated at pH 12.5 (Fig. 6). Again, this fits with the higher reactivity of the supports activated to the highest pH values.

Next, we inactivated the biocatalysts at pH 5, 7 and 9 after blocking them with Gly (optimal blocking agent) [82] (Fig. 7). While at pH 5 and 7 the immobilized trypsin stability was similar (Fig. 7), pH 9, the stability of immobilized trypsin was smaller than at the other pH values and we could not use 72 °C. That way the inactivation temperature at this pH was fixed at 60 °C.

Although differences in the biocatalysts stabilities were short, in all cases, the highest stability was observed using the support prepared at pH 11.5 for 2 h. This support presented similar reactivity with ethylamine than the support prepared at pH 11.5 for 6 h, the N/S ratio decreased, suggesting the presence of non-reactive vinyl sulfone groups. The stability of the preparation on the support prepared for 6 h at pH 11.5 or the support prepared at pH 12.5 for 30 min are very similar in all instances. The lowest stability was obtained using the support prepared at pH 10.5(Fig. 7). This support presented the lowest reactivity versus ethylamine and not good N/S ratio after ethylamine modification (Fig. 4).

The results suggested that not only the amount of reactive vinyl sulfone groups is relevant in defining the stability of immobilized trypsin, but also the N/S ratio is important in the final stability. The unreactive vinyl sulfone groups seem to play a negative role on the stability of the enzyme, although they do not affect the enzyme immobilization rate. This may be expected; if they are caused by polymerization on the vinyl groups, even giving the same amount of reactive groups, increasing the spacer arm length has a negative effect on the enzyme stabilization, as explained in the introduction. If they just are open on the support, the increase the hydrophobicity of the support, making their modification them during the blocking step impossible. Very likely, considering the polymerization of the free divinyl sulfone, the polymerization may be the likeliest explanation, as the vinyl support remains with similar reactivity even in incubation at pH 12.5.

From these results, the activation of agarose at pH 11.5 using 360 mM of divinyl sulfone for 2 h seems to produce the best support for enzyme immobilization/stabilization. However, under these conditions a large percentage of the vinyl sulfone groups are in a non-reactive form. Considering that glyoxyl supports modified with ethylenediamine gave a concentration of N in the support of 3.2 %, the values found in the paper after modification of the vinyl sulfone modification with ethylamine are very similar (considering that ethylendiamine has 2 N per

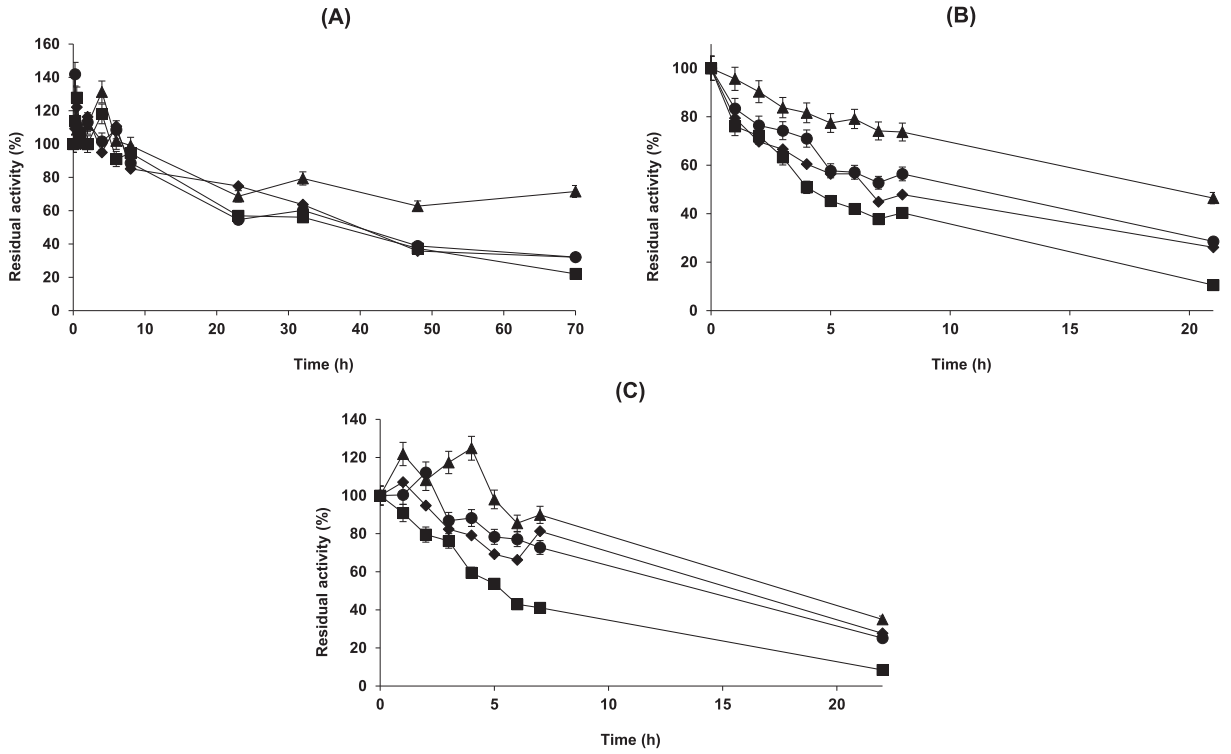

Fig. 6. Immobilization courses of trypsin in different vinyl sulfone supports. (A) pH 10.5 24 h, (B) pH 11.5 2 h, (C) pH 11.5 6 h and (D) pH 12.5 0.5 h. Solid squares: suspension; solid triangles: supernatant; and empty circles: free enzyme reference.

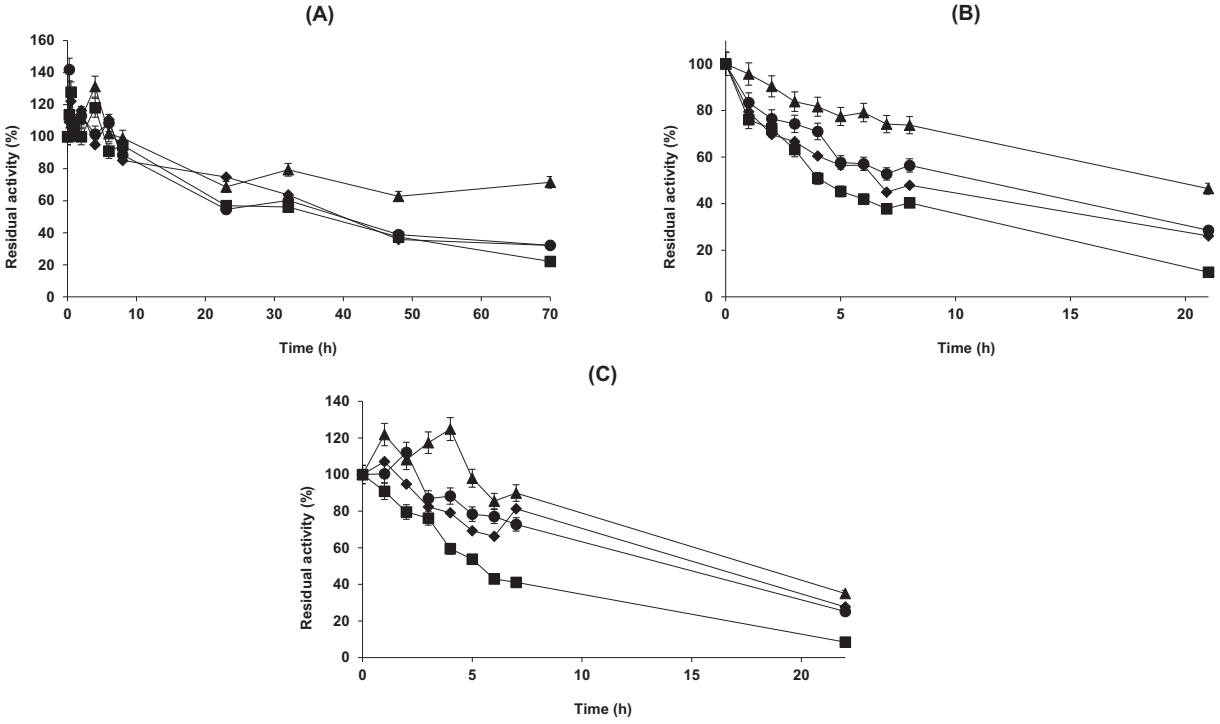

Fig. 7. Thermal inactivation courses of trypsin biocatalysts at different pHs. (A) Sodium acetate pH 5 72 °C, (B) sodium phosphate pH 7 72 °C, and (C) sodium carbonate pH 9 60 °C. Squares: DVS pH 10.5; triangles: DVS pH 11.5 (2 h); circles: DVS pH 11.5 (6 h); diamonds: DVS pH 12.5.

molecule). That means that the decrease of S/N ratio is very likely due more to vinyl sulfone polymerization than to the destruction of active vinyl sulfone.

4. Conclusions

The incorporation of vinyl sulfone groups on agarose beads when incubated with divinyl sulfone improves under alkaline pH value, but there a significant percentage of introduced vinyl sulfone groups that cannot react with ethylamine. The ratio of introduced vinyl sulfone groups and reactive vinyl sulfone groups seems to have some impact on the final support performance in enzyme stabilization. The results point that while the amount of vinyl sulfone in the support is critical for enzyme immobilization rate and stabilization, the presence of non-reactive vinyl sulfone groups presented a negative effect on enzyme stability, not very strong but relevant enough. This fits with the possibility of polymerization of vinyl sulfone groups in the support with a free divinyl sulfone molecules this provides a longer spacer arm but also increase the possibility of having more enzyme-support bonds (and this fits the behaviour of free divinyl sulfone). Considering that the amount of groups is similar using MANEA support and the vinyl sulfone supports activated with ethylamine, this explanation becomes more evident. However, the possibility that some vinyl sulfone groups are just opened by reaction with water at alkaline pH values cannot be discarded. The presence of non-reactive vinyl sulfone could have some effects on enzyme stability, although not in immobilization rate.

From the results, we suggest that activation of agarose beads at pH 11.5 for 120 min using 360 mM divinyl sulfone may be the conditions that give the best support activation, shortly followed by activation at pH 12.5 for 30 min. Obviously, it should be of a great interest to control the incorporation of vinyl sulfone groups to can have a N/S ratio after ethylamine modification of 1, but also lower if for any reason longer spacer arms are desired.

CRediT authorship contribution statement

Pedro Abellanas-Perez: Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation. **Diandra de Andrades:** Writing – review & editing, Writing – original draft, Formal analysis, Data curation, Conceptualization. **Andrés R. Alcántara:** Writing – original draft, Investigation, Formal analysis, Data curation. **Maria de Lourdes Teixeira de Moraes Polizeli:** Writing – review & editing, Formal analysis. **Javier Rocha-Martin:** Writing – review & editing, Writing – original draft, Supervision, Formal analysis, Data curation, Conceptualization. **Roberto Fernandez-Lafuente:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Formal analysis, Data curation, Conceptualization.

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.

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