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Title: BOVINE TRYPSIN IMMOBILIZATION ON AGAROSE ACTIVATED WITH DIVINYLSULFONE: IMPROVED ACTIVITY AND STABILITY VIA MULTIPOINT COVALENT ATTACHMENT

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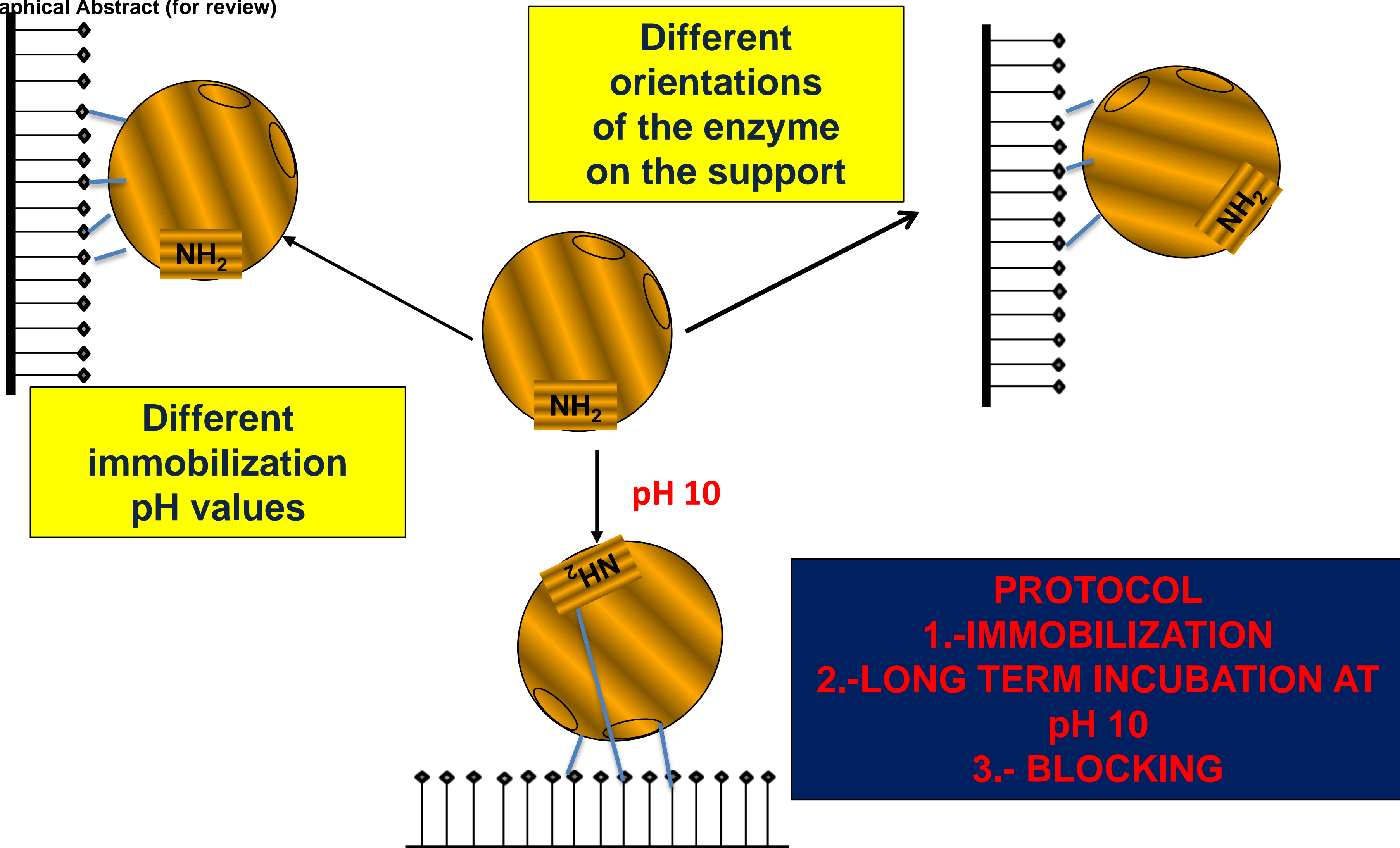
*Graphical Abstract (for review)



TRYPSIN HYPERACTIVATION AND STABILIZATION VIA MULTIPOINT COVALENT ATTAHCMENT

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1 Highlights

2 .Trypsin can be immobilized on DVS –agarose at pH 5, 7 or 10.

3 .Immobilization at pH 10 greatly improves enzyme activity.

4 .72 h alkaline incubation and blocking with EDA improves enzyme stability

5 . At least, an average of 13 aminoacids/molecule are involved in the attachment

6 . This support seems very adequate to get an intense MCA

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7 **BOVINE TRYPSIN IMMOBILIZATION ON AGAROSE ACTIVATED WITH**
8 **DIVINYLSULFONE: IMPROVED ACTIVITY AND STABILITY VIA MULTIPOINT**
9 **COVALENT ATTACHMENT**

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Abstract

Trypsin has been immobilized on divinyl sulfone (DVS) activated agarose at pH 5, 7 and 10. While at pH 5 and 7 immobilization was slow and presented a negative effect on enzyme activity, the immobilization at pH 10 produced a significant increment of activity (by a 24 fold factor). Using this preparation, the effect on enzyme activity/stability of different blocking reagents (used as an enzyme-support reaction end point) were evaluated, selecting ethylenediamine (EDA) because it produced an increase in enzyme activity (a 4 fold factor) and the best results in terms of stability. Next, the effect of alkaline incubation on enzyme activity/stability before the blocking step was analyzed. Activity decreased by 40% after 72 h (but it should be considered that previously it had increased by a 24 fold factor), but the stability significantly improved after this incubation. Thus, after immobilization at different pH values, the immobilized trypsin was submitted to 72 h of alkaline incubation and blocked with EDA. The most active and stable preparation was that immobilized at pH 10. This preparation was less stable than the glyoxyl preparation in thermal inactivations (by less than a twofold factor), but was more stable in organic solvent inactivation (also by less than a twofold factor). The number of groups involved in the enzyme support attachment was 6 Lys using glyoxyl and became a minimum of 13 (including Lys, Tyr and His) using the DVS-activated support (the precision of the method did not permit to analyze the implication of some of the 3 terminal amino groups). Thus, this DVS-agarose support seems to be a very promising support to permit a very intense enzyme-support multipoint covalent attachment.

Keywords: enzyme immobilization stabilization, multipoint covalent attachment, trypsin, divinylsulfone, enzyme hyperactivation, immobilization optimization

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50 1. **INTRODUCTION**

51 Divinylsulfone (DVS) activated supports have been used to immobilize different
52 enzymes and proteins [1–9]. Recently, DVS activated agarose beads have been proposed as a
53 support suitable to stabilize enzymes via multipoint covalent attachment [10]. Vinylsulfone
54 groups can react with different moieties of aminoacids of proteins without any previous
55 activation of the protein (Lys, Tyr, His, terminal amino group(s)) [10]. The enzyme-support
56 reaction produces stable one point bonds after immobilization, not being necessary to stabilize
57 them to avoid the bond breakage during operation even under the most drastic conditions [10].
58 Nevertheless, it was also convenient, as reaction end point, to block the remaining vinylsulfone
59 groups by using different nucleophiles (e.g., aminoacids, amino or thiol compounds) to prevent
60 enzyme-support uncontrolled reactions [10]. The reactive groups are also very stable in storage
61 and immobilization conditions, and are able to immobilize proteins in a broad range of pH
62 values (in [10] chymotrypsin was immobilized at pH values from 5 to 10), although at alkaline
63 pH value the immobilization is faster. The further long term incubation of the immobilized
64 chymotrypsin at alkaline pH values enabled the achievement of higher stabilization factors, even
65 surpassing the results obtained with glyoxyl agarose [11], via multipoint covalent attachment
66 [10].

67 Compared to other supports that have been successfully employed to stabilize enzymes
68 via multipoint covalent attachment (epoxy [12-14], glutaraldehyde [15] or glyoxyl [16]
69 activated supports), DVS activated supports offer some advantages.

70 For example, epoxy and DVS activated supports can react with the same range of groups
71 (epoxy groups can also react with carboxylic moieties, but very slowly) [17]. However, DVS-
72 agarose is much more reactive than epoxy, they can directly immobilize enzymes via covalent
73 attachment [10], while the epoxy groups require the previous adsorption of the enzyme on the
74 support surface [18].

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75 Compared to glyoxyl supports, the main advantage of DVS supports to produce an
76 intense multipoint covalent attachment is that the support may react with other groups different
77 to primary amino groups as it occurs using glyoxyl supports[19]. Moreover, just thinking in a
78 support to immobilize biomacromolecules and not to stabilize them, it is possible to use lowly
79 activated supports (the unstable imine bonds of glyoxyl-supports make the use of relative highly
80 activated supports necessary even to get the enzyme immobilization) [19] and furthermore it is
81 not necessary to perform the immobilization at alkaline pH value, nor is it necessary to use a
82 reduction step (e.g., using sodium borohydride) like when using glyoxyl supports [20]. The
83 main drawback is that the spacer arm is longer (to the agarose -CH_2OH groups, 4 C atoms and 1
84 S must be added (see scheme 1)). And that may reduce the rigidification achieved by the
85 multipoint covalent attachment [21–24]. However, this longer spacer arm may also permit to
86 involve a larger percentage of the enzyme surface in the enzyme-support reaction[21–24].

87 The low reactivity of epoxy and glyoxyl groups under certain circumstances has
88 permitted the development of heterofunctional support to immobilize enzymes via different
89 orientations promoted by other groups (immobilized metals, ionic groups) but via the same
90 chemistry and under the same immobilization conditions [25]. DVS-supports may be too
91 reactive with enzymes to use this strategy to direct the enzyme immobilization, as they can
92 covalently immobilize proteins in the range of pH 5-10. However, the reactivity of the different
93 enzyme groups at different pH values is different, and this may permit to alter the enzyme
94 orientation just altering the immobilization pH [10].

95 In this new paper, DVS-agarose support has been used to immobilize one of the most
96 utilized proteases, trypsin [26]. The properties of this immobilized enzyme will be compared to
97 those obtained via immobilization in glyoxyl support (a very stable preparation) [27,28] that has
98 been utilized in different protein hydrolytic processes [29–32].

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100 **2. Materials and methods**

101 **2.1. Materials**

102 Divinylsulfone, bovine trypsin (E.C. 3.4.21.4), benzoyl-arginine *p*-nitroanilide (BANA),
103 ethylenediamine (EDA), ethanolamine, glycine (Gly), aspartic acid (Asp), and 2-
104 mercaptoethanol were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Agarose
105 beads 4BCL support was purchased from Agarose Bead Technologies (ABT), Spain. All other
106 reagents were of analytical grade.

107 All experiments were performed by triplicate and the results are reported as the mean of
108 this value and the standard deviation (usually under 10%).

109

110 **2.2. Enzymatic assays**

111 The activity of the soluble or suspended enzyme (10 mg/mL) was assayed by
112 determination of the increase in absorbance at 405 nm which accompanies the hydrolysis of the
113 synthetic substrate BANA (100 or 200µL soluble or suspended enzyme were added to 2.5mL of
114 50mM sodium phosphate containing 30% (v/v) ethanol at pH 7 having 2mM BANA, at 25ºC
115 [28].

116

117 **2.3. Preparation of glyoxyl-support**

118 The activation of agarose gels was performed according to the procedure described in
119 [19] the gel was suspended in 1M NaOH and 0.5 M $NaBH_4$ 2:1 (v/v). These reducing conditions
120 prevent oxidation of the gel. While keeping this mixture in an ice bucket, glycidol was added

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121 dropwise in order to reach a 2 M final concentration. The resulting suspension was gently stirred
122 overnight at room temperature. The modified gel was then washed once with abundant distilled
123 water (pH 7), incubated in an aqueous solution (300 mL) containing 60 µmoles $NaIO_4$/g gel in
124 order to achieve glyoxyl groups. This oxidative reaction was allowed to proceed for 2–3 h under
125 mild stirring at room temperature [33]. Then, the support was washed with distilled water and
126 stored at 4ºC.

127

128 **2.4. Preparation of divinylsulfone-support**

129 A volume of 7.5 mL of divinylsulfone was stirred in 200 mL of 333 mM sodium
130 carbonate at pH 12.5 until the medium becomes homogeneous [10]. Then a mass of 10 g of
131 agarose beads was added and left under gentle agitation for 35 minutes. Finally, the support was
132 washed with an excess of distilled water and stored at 4°C.

133

134 **2.5. Immobilization of trypsin on the different supports**

135 **2.5.1. Immobilization on glyoxyl-support**

136 A 10 g portion of support was suspended in 100 mL of trypsin solution (10 mg protein/g
137 support) in 50 mM sodium carbonate at pH 10 and 25°C for 72h [27]. Derivatives were then
138 reduced by addition of solid $NaBH_4$ (0.1% (m/v))[20]. After gentle stirring for 30 min at room
139 temperature, the resulting derivatives were washed with abundant distilled water to eliminate
140 residual sodium borohydride.

141

142 **2.5.2. Immobilization on divinylsulfone-support**

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143 A 10 g portion of support was suspended in 100 mL of solutions (10 mg/g) of trypsin in
144 10 mM sodium acetate at pH 5, sodium phosphate at pH 7 or sodium carbonate at pH 10 at 25
145 °C. After the complete immobilization of the enzyme, the immobilized enzyme derivatives were
146 filtered and a portion of the derivatives was incubated in 100 mL of 100 mM bicarbonate at pH
147 10.0 and 25°C for 72 h. As a reaction end point , the DVS preparations were incubated in 1M
148 EDA at pH 10 and 25ºC for 24h to block the remaining reactive groups (this was the optimal
149 blocking reagent using chymotrypsin and this support) as a reaction end-point [10]. Finally, the
150 immobilized preparations were washed with an excess of distilled water and stored at 4°C.

151

152 **2.6. Thermal inactivation**

153 To check the stability of enzyme derivatives, 1 g of immobilized enzyme was
154 suspended in 5 mL of 10 mM of sodium acetate at pH 5, sodium phosphate at pH 7 or sodium
155 carbonate at pH 9 at different temperatures. Periodically, samples were withdrawn and the
156 activity was measured using BANA. Half-lives were calculated from the observed inactivation
157 courses.

158

159 **2.7. Enzyme inactivation in the presence of organic solvents**

160 Enzyme preparations were incubated in mixtures of 70% dioxane / 30% of 100 mM
161 Tris-HCl buffer (V/V) at pH 7 at different temperatures. Samples of the suspension were
162 withdrawn periodically, and the enzyme activity was analyzed as described above. The presence
163 of a very small amount of organic cosolvent (approx. 50 μL) during the activity assays did not
164 affect the observed enzyme reaction rates during activity determinations assays. Stabilization
165 was calculated as the ratio between the half-lives of the biocatalyst under study. The
166 experiments were carried out by triplicate and error was never over 10%.

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168 **2.8. Determination of the aminoacids involved on the enzyme-support multipoint covalent**
169 **attachment.**

170 The bonds formed by the enzymes and the glyoxyl (after reduction), or the DVS
171 supports are highly stable and may stand acid hydrolysis of proteins. This strategy has been
172 used previously with very good results on different glyoxyl-immobilized enzymes[27,28]. The
173 number of free amino acids of the different biocatalysts was obtained by determining the amino
174 acids of each biocatalyst that could be released to the medium by the method previously
175 described by Alaiz et al. [34]. Briefly, samples of each derivative, containing 2-4 mg of enzyme,
176 were hydrolyzed with 6 N HCl at 120ºC and subsequently analyzed by high-performance liquid
177 chromatography (HPLC) after derivatization with ethoxymethylenmalonate, using D,L-α-
178 aminobutyric acid as internal standard, and a 300 × 3.9 mm i.d. reverse-phase column
179 (Novapack C18, 4 μm; Waters). Likewise, amino acid composition of soluble trypsin was also
180 determined, in the presence and in the absence of blocked DVS support to ensure the lack of
181 artifacts caused by the support. Concentrations (mol/g protein) of each amino acid in samples
182 were determined and the number of residues was calculated as followed:
183 Number of amino acid/molecule of trypsin × amino acid concentration in sample/ amino acid
184 concentration in trypsin.
185

186 **2.9. Studies of enzyme structure and aminoacid accessibility**

187 Protein structures were modeled using PyMol software version 0.99 [35]. Surface
188 accessibility (ASA) values of residues from 2PTN were calculated by the web-based program
189 ASA-view [36–38].

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190

191 **3. Results**

192 **3.1 Enzyme structure**

193 **Table 1** shows medium accessibility of the trypsin aminoacids that can react with DVS.
194 The enzyme has 3 terminal amino groups, the Ile 16 (very poorly exposed), the Asp 194 (not
195 exposed at all) and the Ser 146 (exposed). It has also 14 Lys, all of them with some degree of
196 exposition, 10 Tyr (only 3 with very low medium exposition), 3 His with a moderate exposition
197 and 12 Cys, all of them involved in disulfide bridges (Cys 22-Cys 157; Cys 42-Cys 58; Cys 128-
198 Cys 232; Cys 136-Cys 201, Cys 168-Cys-182, Cys 191-Cys 220), only Cys 128 has a moderate
199 exposition. Using chymotrypsin, some Cys were involved in the final enzyme-support
200 attachment, suggesting that some disulfide bonds could be broken during the enzyme-support
201 reaction (perhaps due to the alkaline pH, the distortion generated by the attachments, etc).
202 **Figure 1** shows the model of the trypsin structure, with all the reactive groups marked.

203

204 **3.2 Immobilization of trypsin on DVS-agarose**

205 **Figure 2** shows the immobilization of trypsin on DVS-agarose. The immobilization rate
206 increased when the pH increased. Thus, only around 20% of the enzyme was immobilized after
207 24 h at pH 5, while at pH 7 the immobilization yield reached a value of 50% (**Figure 2a**). In
208 contrast, the immobilization at pH 10 was complete in just 30 minutes. These results agreed
209 with the reactivity described for the different groups versus DVS, and also with the
210 immobilization rate found using chymotrypsin [10]. Looking at the activity, a much unexpected
211 result is observed: the activity increase when trypsin is immobilized at pH 10 (**Figure 2b**), and
212 the activity becomes multiplied by 24 after 3 h. The immobilization at pH 7 and 5 produced a
213 decrease in enzyme activity (by around 40-50%). These results suggested that the

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214 immobilization of trypsin on DVS support produced some conformational changes, that in the
215 case of the immobilization at pH 10 are positive while if the immobilization is performed at pH
216 5 or 7, the changes (even if less reaction between the enzyme and the support may be expected)
217 are negative. This may be explained if the immobilization is via different areas when the pH is
218 lowered, and that may occur because different protein groups may be more or less relevant for
219 the immobilization depending on the immobilization pH [10].

220

221 **3.3 Effect of the blocking reagent on the enzyme activity/stability**

222 As we have explained before, the blocking of the support after immobilization is
223 convenient after immobilization to prevent any undesired enzyme-support reaction [10]. This
224 blocking may be performed with a variety of different nucleophiles [10], and we have assayed
225 these reagents for the DVS-Trypsin after immobilization at pH 10. **Figure 3** shows the effect on
226 enzyme activity of the blocking reagent with Gly (2.5 folds) and EDA (4 folds) produced an
227 increase on enzyme activity, while mercaptoethanol produced a severe decrease in enzyme
228 activity (perhaps because of the disulfide bridges breakage) and Asp or ethanolamine has not a
229 very significant effect on enzyme activity. Checking the stability at pH 7 and 70ºC (results not
230 shown), the best blocking is EDA, with a half-live of 25 min, while the worst is
231 mercaptoethanol, with a half-live of 5 minutes (this result agrees with a likely breakage of the
232 disulfide bonds). Glycine blocking gives an enzyme stability quite poor (half-live of 7 minutes).

233 Thus, considering together stability and activity parameters, EDA has been selected as
234 blocking reagent of DVS-Trypsin preparations in all further studies.

235

236 **3.4 Effect of the long term incubation at alkaline pH values on Trypsin-DVS preparations**
237 **activity/stability**

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238 Multipoint covalent attachment has been reported to be a complex and slow process,
239 which requires time and pH conditions that may increase the enzyme/support reactivity [10,39].
240 Thus, the enzyme after immobilization at pH 10 and before blocking of the support was left to
241 react with the support for 24 or 72 h. **Figure 4a** shows the activity evolution during this time (a
242 decrease to around 60%) while the stability of the enzyme increased in a very significant way
243 (by a 6 fold factor) (**Figure 4b**). Thus, an incubation of the immobilized enzyme at pH 10 for 72
244 h was selected as a good condition set to get an intense multipoint covalent attachment.

245

246 **3.5 Comparison of the different DVS-trypsin preparations**

247 Trypsin immobilized at pH 5, 7 or 10 on DVS support were incubated at pH 10 for 72 h.
248 This time under alkaline pH should be enough to equal any initial difference on the number of
249 enzyme-support bonds, if differences are detected in enzyme properties they should be caused
250 by a difference in the immobilization step (likely, a different enzyme orientation regarding the
251 support surface), not in the possibilities of enzyme-support reaction. Thus, 3 different DVS-
252 Trypsin preparations were elaborated and their properties were compared. **Table 2** shows the
253 activity recovery for each preparation and their thermal and organic solvent stability. The
254 alkaline incubation produced an increased in thermal stability of immobilized trypsin under all
255 conditions, and the most stable preparation is always the enzyme immobilized at pH 10, even
256 though this exhibited a hyperactivated enzyme conformation among the long term incubated
257 preparations, the less stable is that immobilized at pH 5 **(Table 2).**

258 Inactivations in 70% (V/V) dioxane solvent gave a similar picture. The most stable
259 preparations are those incubated at pH 10 for 72 h. Among this, the most stable preparation is
260 that immobilized at pH 10 for 72 h, while the least stable preparation is that immobilized at pH
261 5, although differences are shorter than in thermal inactivations **(Table 2)**.

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263 **3.6 Comparison between different preparations of immobilized trypsin**

264 **Figure 5** shows the inactivation courses of the most stable DVS-preparation (that
265 immobilized at pH 10 and incubated for 72 h) and CNBr and glyoxyl-trypsin in both, thermal
266 inactivations and organic solvent. The CNBr-trypsin is the enzyme preparation with a lower
267 stability; activity is fully destroyed in the first activity measure. The thermal stability of the new
268 preparation is under the one prepared with glyoxyl; in any case the difference is not very large
269 (more than a two-fold factor, a half-live of 25 versus 60 minutes) (**Figure 5a).** It should be
270 considered that glyoxyl-trypsin has been reported as a very stable preparation in the literature (
271 Pedroche et al., 2007). The inactivation in organic solvents offers a different situation. Now the
272 hyperactivated DVS-trypsin is more stable than the glyoxyl agarose (half-live of 80 min versus
273 30 minutes) (**Figure 5b**).

274 To detect the number of aminoacids involved in the immobilization, the preparations
275 were submitted to acid hydrolysis and aminoacids not involved in the immobilization were
276 analyzed (**Table 3**). From this study, glyoxyl-trypsin has a minimum of 6 enzyme-support bonds
277 (via Lys), discarding the terminal aminoacids that cannot be detected using this technique (just
278 one amino acid decrease is not enough to have a significant change). The DVS-trypsin had 9
279 Lys involved in the immobilization, but also one His seemed to be involved in the enzyme
280 support attachment and 3 Tyr. None of the Cys seemed to be involved in the enzyme-support
281 reaction, as expected from the implication on disulfide bridges and low exposition, in opposition
282 with the results obtained immobilized chymotrypsin [10]. Thus, the comparison between both
283 preparations is 6 enzyme support bonds using glyoxyl versus 13 using DVS. This support seems
284 to have very good prospects to give very intense multipoint covalent attachment.

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285 The lower thermal stability of the DVS-trypsin when compared to glyoxyl even though
286 the number of enzyme-support bonds is higher may be caused by different reasons. First, it
287 should be considered that the DVS-trypsin has an enzyme hyperactivated form and this form
288 may present a lower stability that the native trypsin preparations. Moreover, the spacer arm is
289 longer now than using glyoxyl supports, thus the rigidification induced per bond may be lower
290 using DVS. Finally, the vinylsulfone spacer arm is moderately hydrophobic, and forming a layer
291 under the immobilized enzyme. The presence of hydrophobic groups around the enzyme has
292 been reported to be negative for enzyme stability [40].

293

294 **4. Conclusions**

295 DVS-activated agarose has been found to be a very useful tool to get immobilized
296 preparations of the enzyme with a very intense multipoint covalent attachment (even 13 residues
297 can be involved in the immobilization of the optimal trypsin preparation). On the other hand, the
298 area of the protein involved on the immobilization seems to be different depending on the pH
299 value. First, the effects of the immobilization on enzyme activity are very different (partial
300 inactivation at pH 5 or 7, a very high hyperactivation at pH 10). Secondly, the effects on
301 stability after 72 h of incubation at pH 10 (that should give to all preparations the same
302 possibilities to have the same multipoint covalent attachment) is dependent on the
303 immobilization pH. The blocking step is also a very relevant point in the immobilization on this
304 support, and it alters not only the stability, but also the activity.

305 The most stable and active preparation was obtained by immobilizing the enzyme on
306 DVS-support at pH 10 for 72 h and blocking the support with EDA, the observed activity was
307 almost 100 fold higher than that of the free enzyme or glyoxyl preparation, while the stability
308 was around half of that of the glyoxyl preparation in thermal inactivations while were more

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309 stable in the presence of organic solvents. The reasons for this increased activity when trypsin is
310 immobilized at pH 10, that we have explained in this paper by positive conformational changes
311 (based in the high number of enzyme support linkages a conformational change is almost sure),
312 are under investigation, trying to shortcut the problems of the support interference to determine
313 the enzyme structural changes.

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394 **Figure legends**

395 **Figure 1. 3D surface structure model of trypsin.** The trypsin structure with the external DVS-
396 reactive groups marked. (Lys, Tyr, His, terminal amino acids). The 3D surface structure was
397 obtained using PyMol version 0.99. The 3D structure of trypsin was obtained from the Protein
398 Data Bank (PDB). For trypsin pdb code is 2PTN.

399

400 **Figure 2. Effect of pH on the immobilization courses of Trypsin on DVS-agarose beads.**
401 Other features are described in Section 2. Panel A: circles, solid black line: suspension pH5;
402 circles, solid dash line: supernatant pH5; Square, solid black line: suspension pH7; Square,
403 dash line: supernatant pH7; Panel B: triangles, solid black line: pH10 suspension; Triangles,
404 dash line: pH10 supernatant.

405

406 **Figure 3. The effect on enzyme activity of the blocking reagent on DVS-immobilized**
407 **trypsin.** Other features are described in Section 2. Circles: Glycine; Squares: Ethanolamine;
408 Triangles: EDA; Rhombus: Mercaptoethanol; Stars: Aspartic acid.

409

410 **Figure 4. Effect of the long term incubation at alkaline pH values before blocking on**
411 **Trypsin-DVS preparations activity/stability.** Other details are described in Section 2. Panel
412 (A): Effect of the incubation at pH 10 of the stability of Trypsin immobilized on DVS-agarose
413 beads. Panel (B): Inactivation courses of immobilized Trypsin on DVS-agarose beads incubated
414 for different times at pH 10 before blocking. The inactivation was performed at pH 7 and 70°C:
415 Square, solid black line: Trypsin-DVS (24h incubated at pH 10, Circles, solid black line:
416 Trypsin-DVS (72h incubated at pH 10);

417

418 **Figure 5. Inactivation courses of different trypsin preparations.** Other features are described
419 in Section 2. Panel (A): Thermal inactivation at 75ºC and pH 8: Circles: Glyoxyl-Trypsin;
420 Triangles: DVS-Trypsin; Rhombus: CNBr-Trypsin. Panel (B): Inactivation course of different
421 Trypsin preparations on 70% dioxane: Circles: Glyoxyl-Trypsin; Triangles: DVS-Trypsin;
422 Rhombus: CNBr-Trypsin.

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424 **Table 1.** List of reactive groups of trypsin and their medium accessibilities (ASA).
425 Calculations have been performed as described in Section 2. ASA values of residues from 2PTN
426 were calculated by the web-based program ASA-view.

427

Aminoacid	% ASA	Aminoacid	% ASA	Aminoacid	% ASA
N-terminal (Ile-16)	1.1	Lys-224	30.6	His-57	36.3
N-terminal (Ser-146)	46.1	Lys-230	29.2	His-91	13.7
N-terminal (Asp-194)	0	Lys-239	53.5	Cys-22	11.8
Lys-60	35	Tyr-20	47.3	Cys-42	4.9
Lys-87	31.1	Tyr-29	10.3	Cys-58	2.1
Lys107	18	Tyr-39	61.8	Cys-128	39.6
Lys-109	65.1	Tyr-59	64.6	Cys-136	2.8
Lys-145	53.5	Tyr-94	27.1	Cys-157	1.4
Lys-156	22.8	Tyr-151	51.9	Cys-168	1.4
Lys-159	50.1	Tyr-172	0.5	Cys-182	0
Lys-169	37.9	Tyr-184	33.7	Cys-191	0
Lys-188	47.2	Tyr-228	0.5	Cys-201	2.1
Lys-204	31.1	Tyr-234	3.3	Cys-220	0
Lys-222	77.3	His-40	20.3	Cys-232	14.6

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430 **Table 2.** Relative activities (after all steps of preparation) and half-lives in different conditions
431 (expressed in minutes) of the different trypsin- DVS-preparations under different inactivation
432 conditions. Experiments were performed as described in Section 2.

433 [a]100 is the activity of the soluble enzyme.

434

Trypsin preparation	Recovered activity (%)[a]	Inactivation conditions (half-lives)			
	100	pH 5, 65 ^{o}C	pH 7, 70 ^{o}C	pH 9, 55 ^{o}C	dioxane 70%
DVS-pH5-pH10-EDA	300	25	19	26	140
DVS-pH7-pH10-EDA	250	41	37	34	142
DVS-pH10-pH10-EDA	6275	62	57	60	180

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437 **Table 3.** Free aminoacids of different immobilized trypsin preparations. Experiments have been

438 performed as described in Section 2.

Aminoacid	Trypsin (sequence)	Trypsin (experimental)	Glyoxyl-Trypsin	DVS-Trypsin
His	**3**	**3.1±0.1**	**2.9±0.1**	**2.3±0.0**
Pro	8	8.3±0.5	8.3±0.4	8.2±0.5
Tyr	**10**	**10.4±0.1**	**9.4±0.1**	**7.1±0.3**
Val	17	16.2±0.7	15.7±0.4	16.1±1.4
Met	2	2.3±0.1	2.2±0.0	1.8±0.1
Cys	**12**	**12.2±0.4**	**10.0±0.0**	**12.5±0.0**
Ile	15	14.6±0.7	14.1±0.1	13.0±0.0
Leu	14	14.0±0.6	13.3±0.0	14.2±0.3
Phe	3	3.3±0.0	3.1±0.0	3.4±0.3
Lys	**14**	**13.6±0.4**	**6.8±0.0**	**4.5±0.0**

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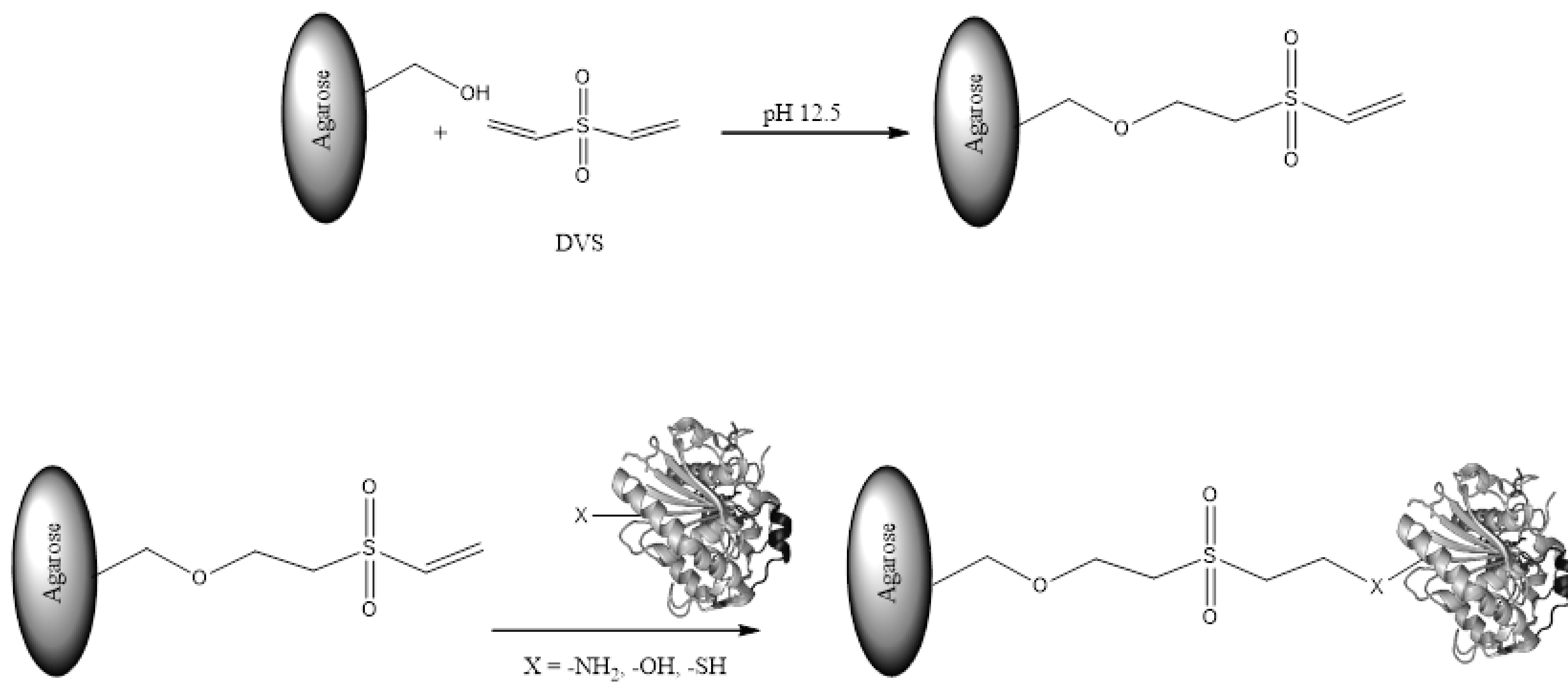
Figure



Scheme 1

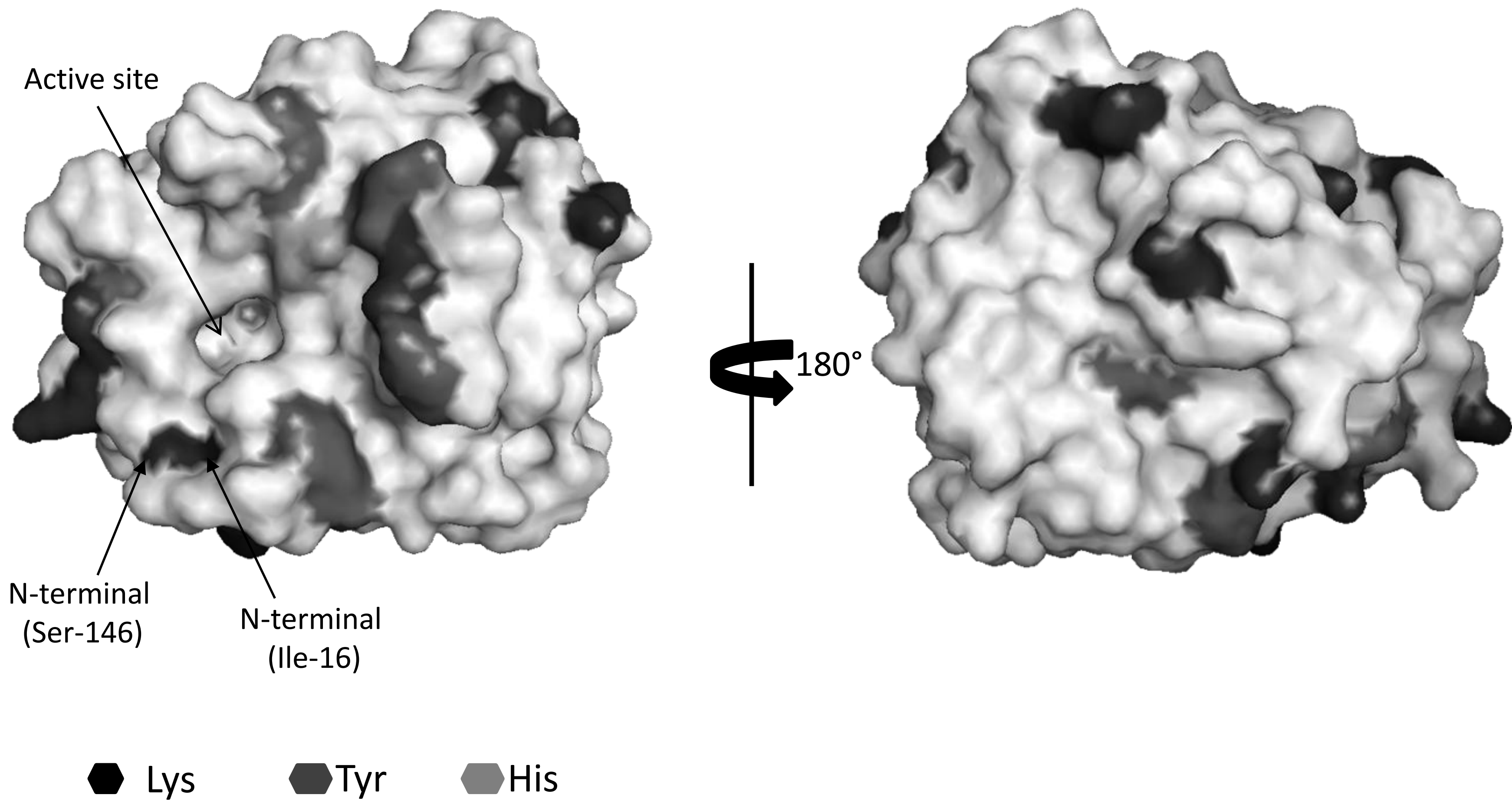


Figure 1

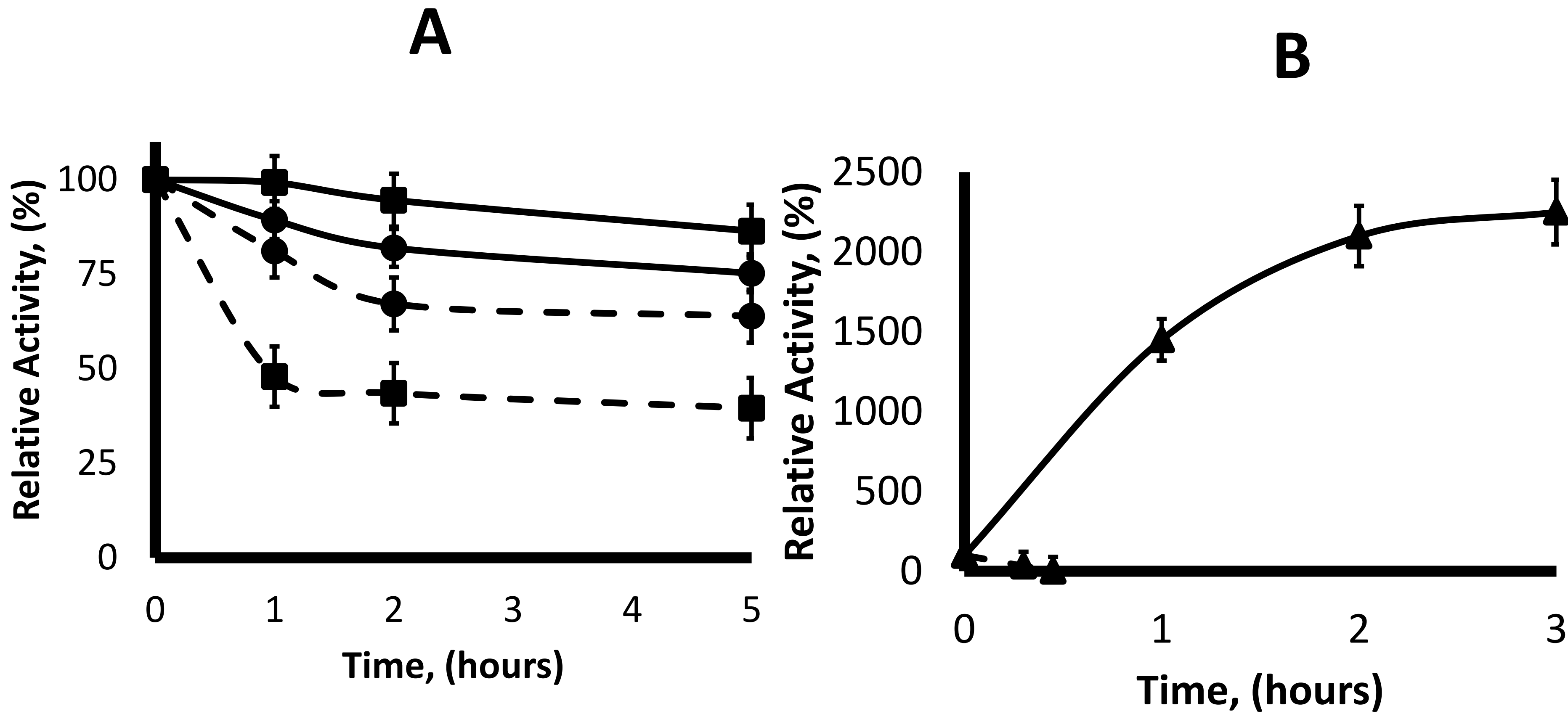


Figure 2

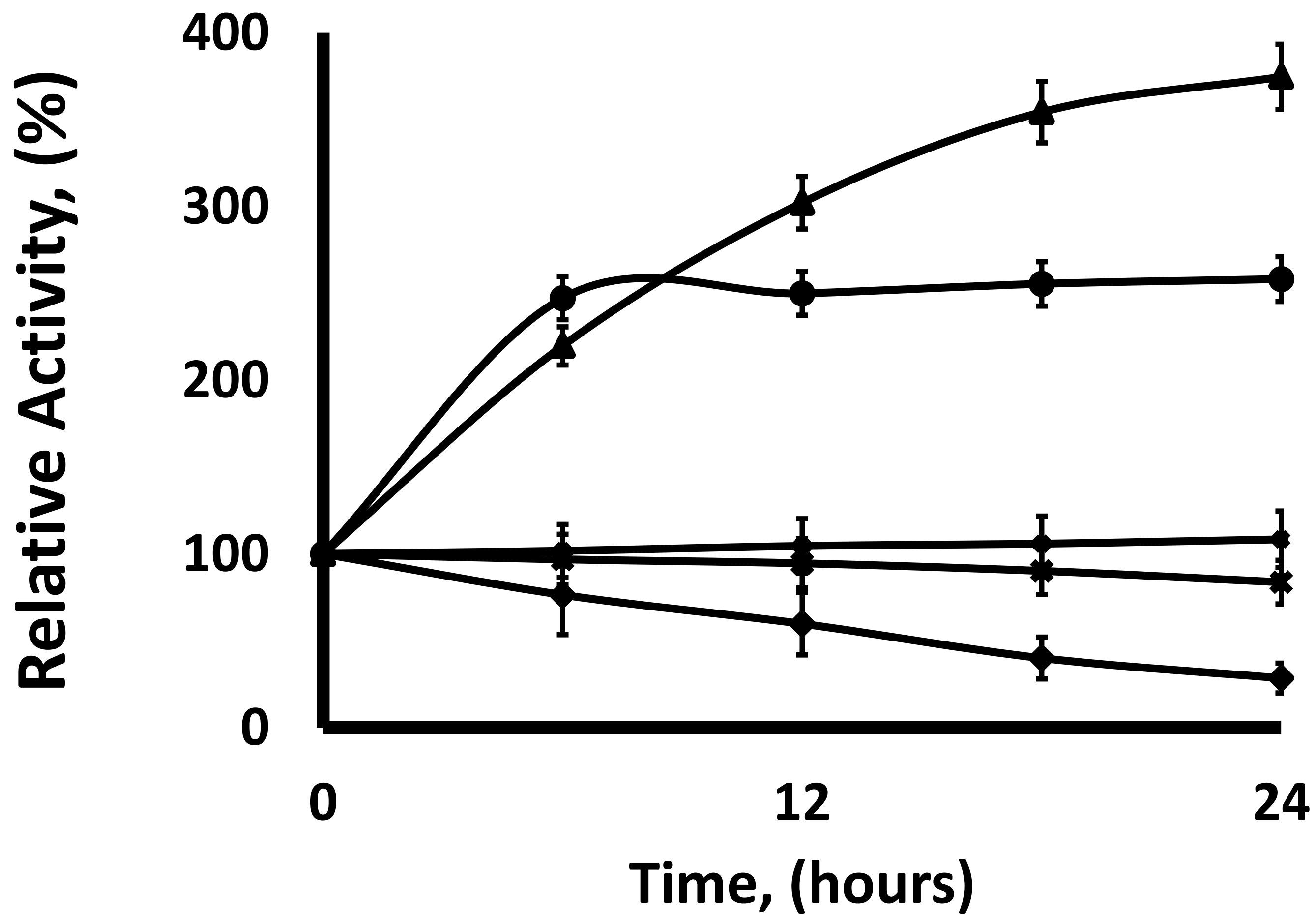
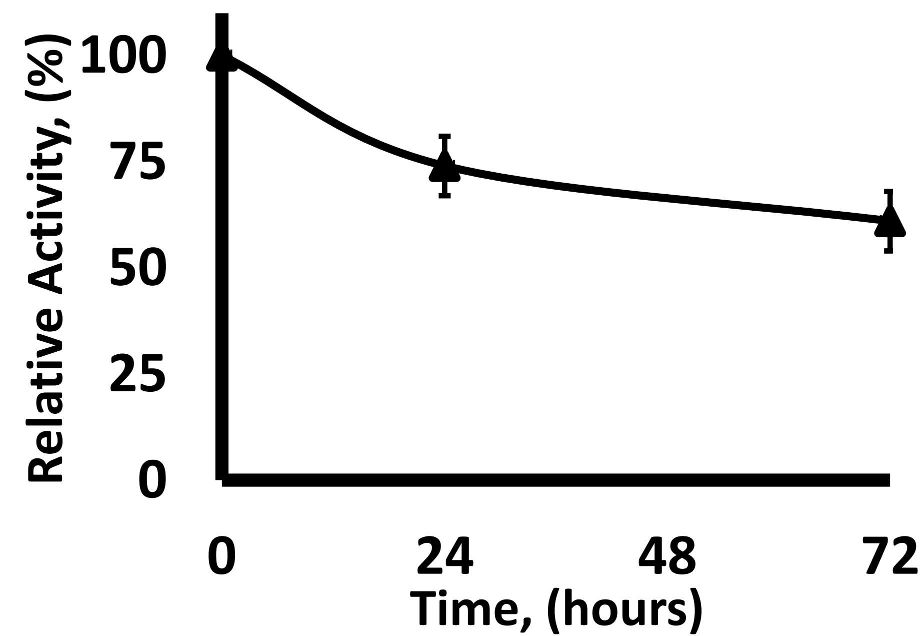


Figure 3



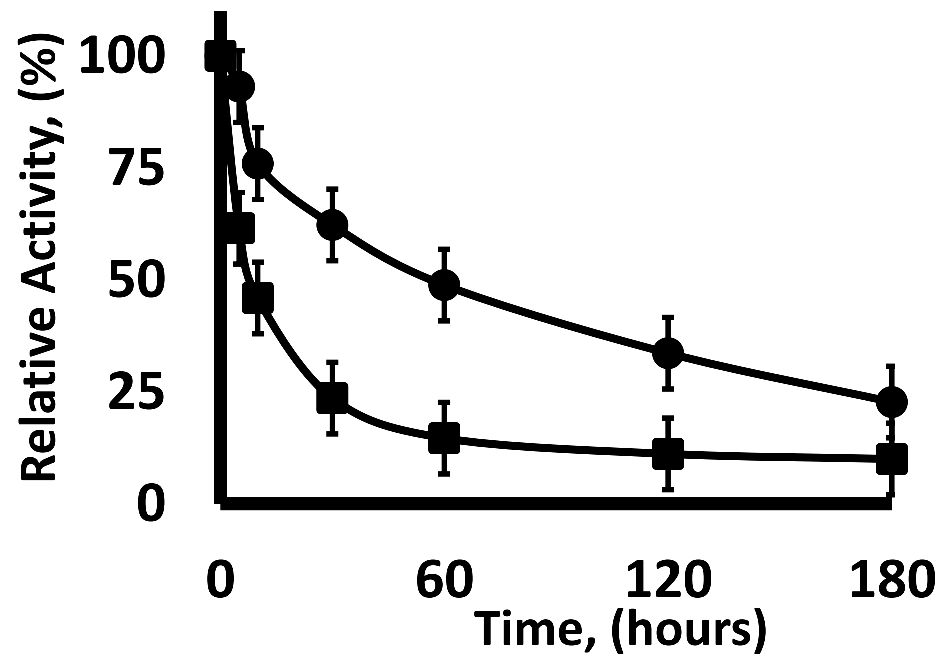
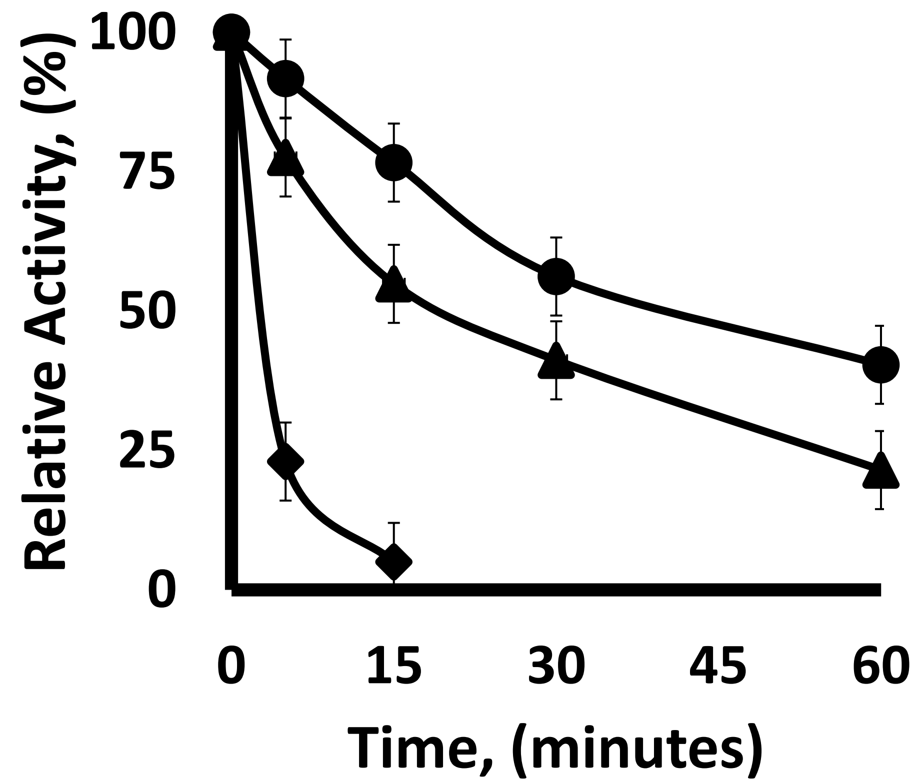


Figure 4



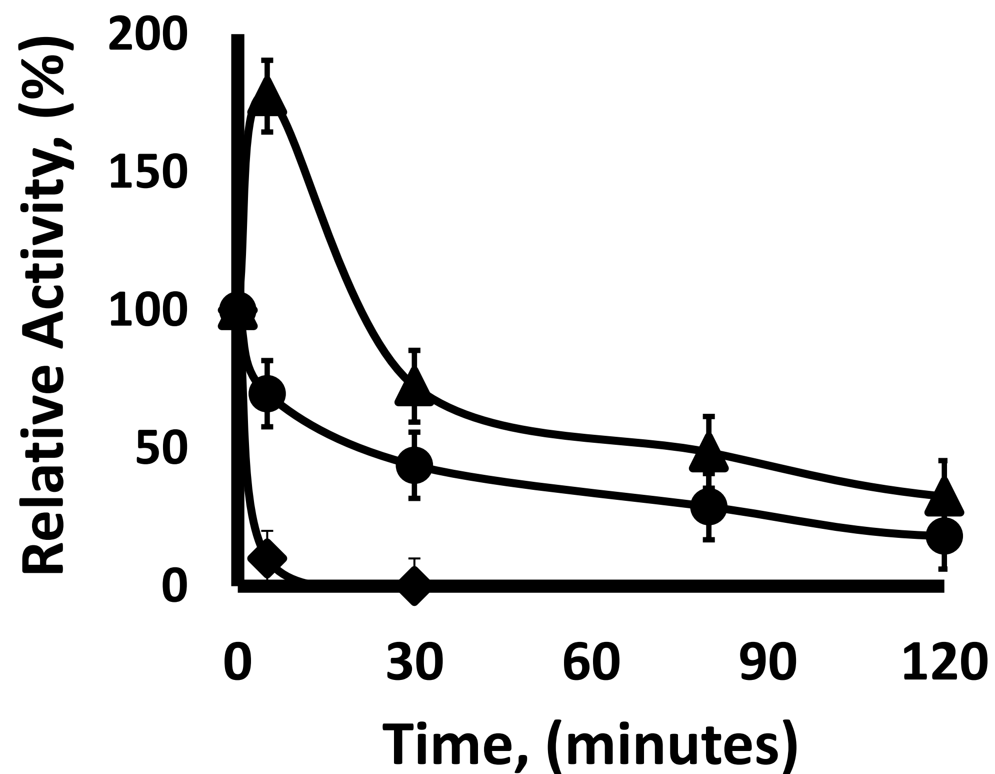


Figure 5

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Scheme 1. Activation of agarose with DVS and the further reaction of the activated support with proteins.

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