IMPROVEMENT OF ENZYME PROPERTIES WITH A TWO-STEP IMMOBILIZATON PROCESS ON NOVEL HETEROFUNCTIONAL SUPPORTS

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Abstract

Novel heterofunctional glyoxyl-agarose supports were prepared. These supports contain a high concentration of groups (such as quaternary ammonium groups, carboxyl groups and metal chelates) that are capable of adsorbing proteins, physically or chemically, at neutral pH as well as a high concentration of glyoxyl groups that are unable to covalently immobilize proteins at neutral pH. By using these supports, a two-step immobilization protocol was developed. In the first step, enzymes were adsorbed at pH 7.0 through adsorption of surface regions which are complementary to the adsorbing groups on the support, and in the second step, the immobilized derivatives were incubated under alkaline conditions to promote an intramolecular multipoint covalent attachment between the glyoxyl groups on the support and the amino groups on the enzyme surface. These new derivatives were compared with those obtained on a monofunctional glyoxyl support at pH 10, in which the region with the greatest amount of lysine residues participates in the first immobilization step. In some cases, multipoint immobilization on heterofunctional supports was much more efficient than what was achieved on the monofunctional support. For example, derivatives of tannase from *Lactobacillus plantarum* on an amino-glyoxyl heterofunctional support were 20-fold more stable than the best derivative on a monofunctional glyoxyl support. Derivatives of lipase from *Geobacillus thermocatenulatus* (BTL2) on the amino-glyoxyl supports were 2-fold more active and 4-fold more enantioselective than the corresponding monofunctional glyoxyl support derivative.

Introduction

The development of simple protocols for the immobilization and stabilization of enzymes is an exciting goal in the field of enzyme biotechnology.[1-6] Immobilization of enzymes by multipoint covalent attachment is one of the most interesting approaches to simultaneously immobilize and stabilize enzymes.[3-4, 6-10] An enzyme molecule that is attached to a rigid support through many covalent linkages via short spacer arms should be highly stabilized. The residues that are involved in the covalent immobilization of the enzyme should maintain their relative positions when conformational changes are caused by different distorting agents, such as heat and organic solvents. Thus, the intensity of the conformational changes caused by these agents should be strongly attenuated.[8, 11-13]

Immobilization of enzymes on highly activated glyoxyl supports under alkaline conditions promotes an orientation on the support surface, in which the enzyme becomes immobilized through its surface region or regions containing the greatest amount of lysine residues. This favorable orientation results in an intense multipoint covalent immobilization [14-15] . In addition to promoting this enzyme orientation, glyoxyl groups have many other properties that result in an intense multipoint covalent immobilization. They are stable under alkaline conditions, they are reactive towards unprotonated primary amines and they do not exhibit steric hindrances for intramolecular reactions.[16] In fact, many industrial enzymes have been stabilized by multipoint covalent attachment on glyoxyl-agarose gels.[9,15]

On the other hand, it would also be interesting to design new immobilization protocols in which multipoint covalent immobilization proceeds through regions of the enzyme that are more sensitive to denaturing agents. For example, these regions could

encompass unstable loops, domains close to the active center and hydrophobic pockets (Scheme 1). Thus, their rigidification could result in a more stable attachment even though the number of covalent links is lower that obtained through linkage with the lysine-rich regions. In addition to stabilization, rigidification of the enzyme through these key regions could also promote a higher recovery of activity after immobilization and an improved selectivity of the immobilized enzymes. that are intended for the modification of non-natural substrates.

Multipoint covalent attachment of enzymes on novel heterofunctional glyoxyl-agarose supports is proposed here. The novel supports contain a high concentration of non-reactive groups that are able to adsorb proteins at neutral pH as well as a high concentration of glyoxyl groups that are unable to covalently immobilize the enzyme at neutral pH. The glyoxyl groups are much more reactive than traditionally-used epoxy-groups. By using these supports, a two-step immobilization protocol was developed. In the first step, enzymes were adsorbed at neutral pH through surface regions that are complementary to the adsorbing groups on the supports, and in the second step, the adsorbed derivatives were incubated under alkaline conditions to promote an intramolecular multipoint covalent attachment between the glyoxyl groups of the support and the primary amino groups close to the adsorbed region of the enzyme (Scheme 2). The preparation of these heterofunctional supports is reported here (Schemes 3 and 4).

The rigidification of several enzymes, incluing penicillin G acylase from *Escherichia coli*, porcine pancreas chymotrypsin, tannase from *Lactobacillus plantarum* and lipase 2 from *Geobacillus thermocatenulatus* (BTL2), using this novel protocol was

studied. The activity, stability and selectivity of the different derivatives were studied and compared to the properties of the derivatives that were obtained with monofunctional glyoxyl supports.

Materials and methods

Materials

α-chymotrypsin type II from bovine pancreas was purchased from Sigma Chem. Co., *G. thermocatenulatus* lipase 2 was expressed in *E. coli* as previously described[17], and tannase strain *L. plantarum* CECT 748T (ATCC 14917, DSMZ 20174), isolated from pickled cabbage was purchased from the Spanish Type Culture Collection. This strain was selected based on its high activity.[18-21] Agarose 10BCL was purchased from Agarose Bead Technologies. Epichlorhydrine, iminodiacetic acid, *p*-aminophenyl boronic acid, triethylamine, mercaptoethanol and sodium metaperyodate were purchased from Sigma Chem. Co. Other reagents were of analytical grade.

Methods

Activation of the supports (Scheme 1):

Activation of agarose with epoxy groups. For this work, 10 g of agarose 10 BCL was suspended in 44 mL water, 16 mL acetone, 3.28 g NaOH, 0.2 g $NaBH_4$ and 11 mL epichlorhydrine. The suspension was stirred mildly for 16 hours and washed with an excess of water. For quantification of the activated epoxy groups, 1 g of the support was treated with 10 mL 0.5 M H_2SO_4 for 2 hours to hydrolyze the epoxy groups. Then, this hydrolyzed support was oxidized with $NaIO_4$, as previously described.[14] The number of epoxy groups was calculated by the difference in periodate consumption between the hydrolyzed support and the initial epoxy support. Periodate consumption was quantified using potassium iodide as previously described.[22]

Modification of agarose supports with different reactive groups. Agarose epoxy-supports were modified with different moieties. In all cases, the ratio of the modifying reactive solution to the support was 1/10 (v/w).

Cationic supports. The epoxy-agarose support was modified with 1 M triethylamine in 50% water/acetone at pH 12 for 24 hours at 25ºC.

Anionic supports. The epoxy-agarose support was treated with 0.5 M iminodiacetic acid at pH 11 for 24 hours at 25ºC.

Metal chelate supports. The anionic supports were modified with a 30 mg/mL solution of 1 of 4 different metallic salts ($CuSO_4$, $NiCl_2$, $ZnCl_2$ and $CoCl_2$) at pH 7.0, 25 ºC for 1 hour.

Boronate supports: The epoxy-agarose support was modified with 5% *p*-aminophenyl boronic acid, dissolved in 20% dioxane at pH 11 for 24 hours at 25ºC.

Monofunctional supports: The epoxy-agarose supports were blocked with 5% mercaptoethanol at pH 8.7 and 25 ºC for 16 hours.

Finally the supports were oxidized with sodium periodate, as previously described.[14]

Enzyme assays:

BTL2 activity test. BTL2 activity was quantified by measuring the increase in absorbance at 348 nm caused by release of p-nitrophenol during hydrolysis of 0.4 mM pNPB. The reaction was performed in 25 mM sodium phosphate at pH 7 and 25 ^{o}C under continuous magnetic stirring and was measured using a thermostatized spectrum. To initialize the reaction, 0.05 mL of a lipase solution or suspension were added to 2.5 mL of the substrate solution. The amount of enzyme or derivative used in the assays yielded a maximum increase in absorbance per minute of 0.15. One international unit (IU) of *p*NPB activity was defined as the amount of enzyme necessary to hydrolyze 1 μmol of *p*NPB per min, under the conditions described above.

Tannase activity test. The esterase activity of tannase was determined using a rhodamine assay specific for gallic acid, as previously described.[23] One unit of tannase activity was defined as the amount of enzyme required to release 1 µmol of gallic acid per minute, under standard reaction conditions.

Chymotrypsine assay. The chymotrypsine assay was performed by recording the increase in absorbance at 405 nm and 25ºC promoted by the release of *p*-nitrophenol produced by the hydrolysis of 0.3 mM of *N*-Benzoyl-*L*-tyrosine *p*-nitroanilide (BTNA) dissolved in 50 mM phosphate in the presence of 40% ethanol at pH 7 during the enzyme-catalyzed reaction. One IU of BTNA activity was defined as the amount of enzyme necessary to hydrolyze 1 µmol of BTNA per minute, under the conditions described above.

Enzyme immobilization:

The enzymes were solubilized in a sodium phosphate buffer (5 mM phosphate for use with ionic supports or 50 mM phosphate for use with boronate and metal-chelate supports), at pH 7 and a temperature of 25^{o}C. Then, 1 g of support (ionic, boronate or metal-chelate) was suspended in 10 mL of the solution, with a maximum enzyme activity of 1 IU/mL. Periodically, samples of the supernatants and suspensions were withdrawn, and the enzyme activity was measured. After the immobilization was complete, the preparations were washed with the phosphate buffer, dried under vacuum and resuspended in 10 mL sodium hydrogen carbonate (5 mM for ionic supports and 50 mM for the other supports) at pH 10 for 3 hours. When non-oxidized supports were used, this alkaline incubation was not performed. Finally, the preparations were reduced by addition of 10 mg sodium borohydride. The metal chelate supports were washed with 5 volumes of 50 mM EDTA at pH 7 before the reduction step. These suspensions were stirred mildly for 30 minutes and then washed with water.

When monofunctional glyoxyl supports were used, the immobilization was performed in a 50 mM sodium bicarbonate solution at pH 10 for at least 3 hours, and they were reduced for 30 minutes using 1 mg/mL sodium borohydride.

Enantioselectivity

The asymmetry of the different BTL2 preparations was measured using mandelyl butyrate as the substrate.[24] For this experiment, 0.5 g of wet immobilized preparations was added to 3 mL of 1 mM substrate in 25 mM sodium phosphate at pH 7 and 25ºC, and then the suspension was stirred mildly. During the reaction, the pH

value was maintained at a constant level by automatic titration using pH-stat. Blank experiments were performed using suspensions of the different matrices without enzyme.

The degree of hydrolysis was followed by reverse-phase high pressure liquid chromatography (HPLC) (Spectra Physics SP 100, coupled with a UV detector Spectra Physics SP 8450) on a Kromasil C18 (25 cm × 0.4 cm) column, supplied by Analisis Vinicos (Spain). Each assay was performed at least in triplicate. The experimental error was under 3%. The elution was performed using a mobile phase composed of acetonitrile (35%) and 10 mM ammonium phosphate buffer (65%) at pH 2.95 at a flow rate of 1.5 mL/min and was monitored by recording the absorbance at 225 nm. The retention time of the butyric acid was 3.7 min, and the retention time of the mandelyl butyrate was 23 min.

At different conversion degrees, the enantiomeric excess (ee_p) of the released acid was analyzed by chiral reverse phase HPLC. The column was a Chiracel OD-R, the mobile phase was an isocratic mixture of 5% acetonitrile and 95% 0.5 M $NaClO_4/HClO_4$ at pH 2.3 and the analyses were performed at a flow rate of 0.5 mL/min by recording the absorbance at 225 nm. The retention time of the *S* isomer was 39 min, and the retention time of the *R* isomer was 42 min. The asymmetry was measured as the ratio of the extent of hydrolysis of the R enantiomer compared to that of the S enantiomer.

Results

New generation of glyoxyl heterofunctional supports.

As discussed in the introduction, supports activated with glyoxyl groups generally are not able to immobilize enzymes at neutral pH. For this reason, we set out to make new supports that are capable of immobilizing proteins via a two step mechanism: adsorption of the enzyme on the support at neutral pH under mild conditions, such as temperature and ionic strength, and incubation at alkaline pH to promote the reaction between the aldehyde groups of the support and the lysine residues of the enzyme. To this end, we propose the use of agarose supports activated with epoxy groups that can be easily functionalized groups that are able to adsorb proteins via different mechanisms.[25] In addition, during activation of the agarose with epoxy groups, a large amount with of diol groups are produced from hydrolysis of some of the epoxide groups, which can then be oxidized with sodium periodate to produce glyoxyl groups. The amount of diol groups was quantified and estimated to be approximately 60% of the total amount of epoxide groups initially activated (Table 1). In this way, the supports would contain a large amount of glyoxyl groups that are capable of establishing intense multipoint covalent linkages, and therefore, a stable immobilized preparation could be obtained.

The mechanism of immobilization on these supports was evaluated using the enzyme BTL2 as a model. For this experiment, the enzyme was added to the monofunctional glyoxyl support at neutral pH, and it was found that immobilization was not promoted (Figure 1). As discussed in the introduction, The glyoxyl supports are generally not capable of immobilizing proteins because the lysine is deprotonated and

reactive only at alkaline pH, and therefore, the required points of attachment cannot be made[14] . In contrast, when the heterofunctional supports were used, immobilization of each of the enzymes was produced within several minutes with the exception of the support activated with carboxylic groups (Figure 1). Moreover, when the enzymes was added to cationic supports at high ionic strength (1 M NaCl), immobilization did not occur. Similarly, immobilization was not measurable when bifunctional chelate-glyoxyl groups were added in the presence of high concentrations of imidazole or when boronate supports were added in the presence of high concentrations of mannitol (data not shown). This result shows that in general, enzymes are not immobilized on these bifunctional supports at neutral pH under conditions at which previous physical adsorption of the enzymes is not promoted. The supports developed in this work, however, allow immobilization of proteins under any conditions, such as mild pH, low or high ionic strength or cold temperatures. In fact, it was shown that in 30 minutes, 80% of the BTL2 was immobilized on the support that was activated with amino-glyoxyl groups, but after being incubated at a high salt concentration, all the immobilized enzyme was desorbed (Table 2). This confirms that the enzyme had only been physically adsorbed on the support. In addition, it was found that with an increase in contact time between the enzyme and support, the amount of released enzyme decreased rapidly. Thus, despite the absence of protein immobilization at neutral pH on monofuntional glyoxyl supports via an intermolecular reaction, the intramolecular protein-glyoxyl support reaction occurs if protein had been previously adsorbed.

The thermal stability of different BTL2 preparations was studied. In the first preparation, the enzyme was adsorbed on amino-glyoxyl supports, in which the glyoxyl

groups were previously reduced; in the second preparation, the enzyme was immobilized on bifunctional amino-glyoxyl supports at pH 8 for 12 hours and in the third preparation, the enzyme was immobilized at neutral pH and incubated for 3 hours at pH 10 and 25ºC. As shown in Figure 2, the most stable derivative was the one that was immobilized on the bifunctional support and incubated at alkaline pH. This may be due to the increased reactivity of the lysine residues after incubation at pH 10. Additionally, it could be seen that the derivative adsorbed on the bifunctional supports at neutral pH was more stable than the one adsorbed on supports with only reactive amine groups. This suggests that even at neutral pH, a certain degree of covalent binding of the enzyme with the aldehyde groups of the support may be promoted.

Once this new generation of glyoxyl-heterofunctional supports was developed, they were used for the immobilization of several enzymes to evaluate different properties of the immobilized derivatives.

Activity of the enzyme preparations immobilized on mono- and heterofunctional glyoxyl supports.

The activities obtained after immobilizing the enzymes on the glyoxyl supports differed depending on the support and the enzyme used in the process. For example, the recovered activity of BTL2 immobilized on monofunctional supports at pH 10 was rather low (approximately 65%). The best result was obtained when the enzyme was immobilized on the heterofunctional amino-glyoxyl support, with 90% of the activity recovered with respect to the activity of the soluble enzyme (Table 3). This suggests

that the type of support on which the enzyme is immobilized could affect the surface regions that are involved in the process.

Stability of the enzyme derivatives immobilized on mono- and heterofunctional glyoxyl supports.

In addition, the stability, against heat or against exposure to organic solvents, of the derivatives obtained after immobilizing the enzymes on the battery of previously synthesized glyoxyl supports was studied. In all cases, each preparation had different stability against the distorting agents. For example, tannase derivatives immobilized on amino-glyoxyl supports were the most stable against temperature and organic co-solvents (figure 3 A and B). Similarly, the most stable chymotrypsin derivative was the one immobilized on boronate-glyoxyl agarose (Figure 4). This is interesting because when the enzymes are immobilized on glyoxyl supports they react through the area that is richest in lysines, which are, in principle, the most rigidifying because of their high reactivity with glyoxyl groups. On the contrary, protein derivatives on bifunctional supports have other places on the surface that can be rigidified; this surface could be the most sensitive to distortion (e.g., containing unstable loops or being close to the active center). This is further evidence of how immobilizing enzymes on different supports rigidifies different regions of the protein, yielding preparations with different stability against factors such as temperature and organic co-solvents.

Enantioselectivity of the enzyme derivatives immobilized on mono- and heterofunctional glyoxyl supports.

The selectivity of the derivatives of BTL2 was also studied, with mandelyl butyrate as a model substrate. The enzyme immobilized on amino-glyoxyl supports exhibited a higher greatest asymmetry which was more than 3 times higher the asymmetry of the derivative from the monofunctional glyoxyl supports (Table 4). This may be further demonstration that the enzyme is immobilized at different regions, allowing the derivatives to have different activity, stability and selectivity.

DISCUSION

Novel heterofunctional glyoxyl supports are easy to prepare. Agarose gels or other hydroxylic supports can be carefully activated with epiclorhydrin. The activated supports have 40% epoxy groups, which are able to react with high concentrations of many small ligands bearing nucleophilic groups, such as amino groups and thiols.[25] The activated supports also have 60% glyceryl groups, which can be converted into glyoxyl groups via periodate oxidation.[14] At first glance, it appears that epiclorhydrin could produce 100% epoxy groups through reaction with all of the hydroxyl groups on the support, but the strong alkaline conditions required for activation promote the hydrolysis of a large number of epoxy groups into glyceryl groups instead.

These novel heterofunctional supports allow the design of novel two-step immobilizations. The first step is a physical adsorption of the enzyme by adsorbing groups on the support surface; the enzyme is immobilized at neutral pH in spite of the

negligible covalent immobilization on the glyoxyl groups. In addition, the enzyme is not immobilized if adsorption is not possible, such as in the presence of high ionic strength for ionic exchange bifunctional supports, in the presence of imidazol for adsorption on metal chelate bifunctional supports or in the presence of glycerol or mannitol for adsorption on boronate bifunctional supports. Boronate groups have been reported to adsorb glycoproteins through their cis-diol moieties;[26-27] however, a crude extract of non-glycosylated proteins from *E. coli* were also able to be adsorbed on boronate supports,[25] perhaps though the involvement of nitrogenated groups of the protein that are different from the α-amino and the trigonal boronate groups.[28]

The subsequent incubation of adsorbed enzymes under alkaline conditions seems to promote an intramolecular covalent immobilization. In fact, enzymes adsorbed via ion exchange are not desorbed at high ionic strength. On the other hand, a longer incubation under more alkaline conditions promotes an increased stabilization of the resulting derivatives. This is a clear indication that intramolecular multipoint covalent attachment was carried out. The stability of each derivative strongly depends on the first adsorption and on the second intramolecular covalent attachment or on the rigidification of the optimal orientation.

All resulting derivatives were more stable than those that had been mildly immobilized on cyanogen bromide-activated agarose (CNBr-activated agarose) and some were more stable than those immobilized on highly activated monofunctional glyoxyl supports at pH 10.0, in which the enzyme is immobilized through the region with the greatest amount of lysine residues, which should produce the most intense multipoint binding.

The most interesting derivatives found in this work were as follows:

a. Chymotrypsin-boronate glyoxyl agarose (BGA) and tannase-amino glyoxyl agarose (AGA) were 2-fold and 20-fold more stable, respectively, than the corresponding monofunctional glyoxyl derivatives (MGA).

b. BTL2-AGA was 2-fold more active than BTL2-MGA in the hydrolysis of mandelyl butirate.

c. BTL2-AGA was 3-fold more enantioselective than BTL2-MGA for the hydrolysis of mandelyl butirate.

A similar strategy was previously proposed to immobilize enzymes on epoxy supports (e.g., Eupergit, Sephabeads). In this case, the use of heterofunctional supports was strictly necessary because of the very low intermolecular reactivity of the epoxy groups with the proteins.[25] In fact, some of the covalent immobilizations described only take place at very high ionic strength on hydrophobic supports. In these cases, an initial hydrophobic adsorption of the enzyme though external hydrophobic pockets is the first event during immobilization.[29] These immobilizations also promote different recovered activity and stabilization. However, these supports do not permit the establishment of very intense multipoint immobilization, as monofunctional epoxy supports do. On the contrary, monofunctional glyoxyl supports directly immobilize proteins under alkaline conditions (e.g., at pH 10). Interestingly, this immobilization occurs in the region of the enzyme having the greatest number of lysine groups, and at first glance, this region may undergo the most intense multipoint covalent

immobilization. In addition, the glyoxyl groups seem to be more suitable for intramolecular multipoint attachment than the epoxy groups, as recently reported.[30]

The combination of the novel orientations reported here and others previously discussed clearly suggest that any enzyme can be covalently multipoint-immobilized via glyoxyl chemistry but with different orientations (see Table 5).

The preparation of this set of enzyme derivatives may allow an improvement of enzyme properties through simple immobilization protocols. This strategy can be applied to any native enzyme without a known 3D structure and without knowing the exact mechanisms of catalytic action and inactivation. Logically, recombinant enzymes with a known 3D structure could be oriented and rigidified in a much more rational way and with very different enzyme orientations.

Conclusions

The design of these two-step immobilization protocols allows us to the perform oriented rigidification of native industrial enzymes and to prepare novel derivatives with different functional properties, such as activity, selectivity and stability. These achievements can be reached without knowing the 3D structure, the catalytic mechanism or the inactivation mechanism of the target enzymes. The first immobilization step has to be an oriented physical adsorption, even a quite slow one, and hence, the additional chemically reactive groups have to exhibit some very special properties. They must be inert in the intermolecular reaction with proteins at neutral

pH, stable under alkaline conditions and suitable for intramolecular reaction with amino groups placed on the surface of the adsorbed enzymes (e.g., absence of steric hindrances for the chemical reaction). Glyoxyl groups, which are not suitable for covalent immobilization of enzymes at pH 7.0, seem to be the most adequate chemically reactive groups for this novel type of immobilization protocol.

Compared to the properties of the strongest multipoint covalently attached derivatives, immobilized on monofunctional glyoxyl groups at pH 10, some interesting results have been here reported for the novel oriented multipoint attached derivatives: improvement of the stability of tannase and chymotrypsin and improvement of the activity and enantioselectivity of a thermostable lipase.

ACNOWLEDGEMENTS

This work has been sponsored by the Spanish Ministry of Science and Innovation (project AGL-2009-07625) and the Comunidad Autónoma de Madrid (Project S0505/PPQ/03449). We gratefully recognize to Spanish Ministry of Science and Innovation for “Ramón y Cajal” contract to Dr. Fernandez-Lorente..

Figure legends

Scheme 1: Rigidification of different regions of enzymes.

Scheme 2: Mechanism of immobilization-stabilization using these new heterofunctional supports.

Scheme 3: Preparation of different heterofunctional glyoxyl supports .

Scheme 4.- Different ligands able to adsorb proteins at neutral pH

Figure 1: Immobilization of BTL2 on heterofunctional glyoxyl supports. Experiments were performed at pH 7 as described in the methods. (♦) Glyoxyl-Cu agarose; (■) boronate glyoxyl agarose; (▲) amino-glyoxyl agarose; (□) carboxy-glyoxyl agarose; (●) monofunctional glyoxyl agarose; (○) amino-glyoxyl agarose in the presence of 1 M NaCl.

Figure 2: Time-courses of thermal inactivation of different BTL2 immobilized derivatives. Derivatives were incubated at 70ºC. (■) BTL2 immobilized on amine supports with previously reduced glyoxyl supports; (▲) BTL2 immobilized on amino-glyoxyl supports at pH 8 for 12 hours; (♦) BTL2 immobilized on amino-glyoxyl supports at pH 8 and incubated for 3 hours at pH 10.

Figure 3: Time-course of inactivation of different tannase derivatives. A) Inactivation was performed in 25 mM sodium phosphate at pH 7 and 55ºC. B) Inactivation was performed at 25ºC in 25 mM sodium phosphate and 30% methanol at

pH 7. (♦) CNBr derivatives ; (▲) monofunctional glyoxyl derivatives; (■) amino-glyoxyl derivatives.

Figure 4: Time-course of thermal inactivation of different immobilized derivatives of chymotrypsin. Inactivation was performed by incubation of the different derivatives in 25 mM sodium phosphate at pH 7 and 70ºC. (♦) Glyoxyl-Cu agarose; (■) boronate glyoxyl agarose; (▲) amino-glyoxyl agarose; (●) monofunctional glyoxyl agarose; (○) soluble enzyme.

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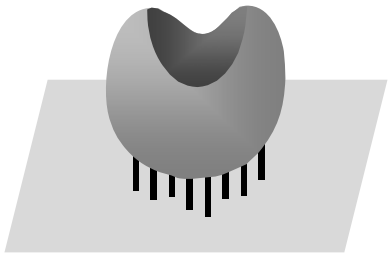
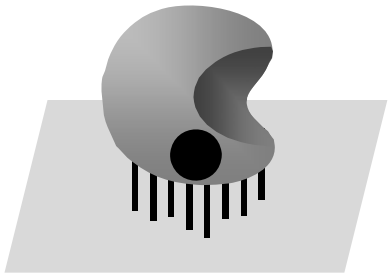
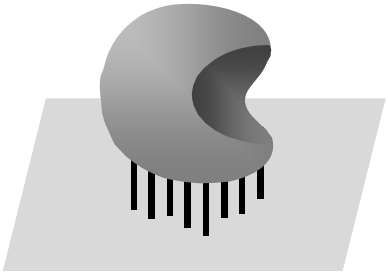
RIGIDIFICATION OF DIFFERENT ENZYME REGIONS



Richest region in lysine

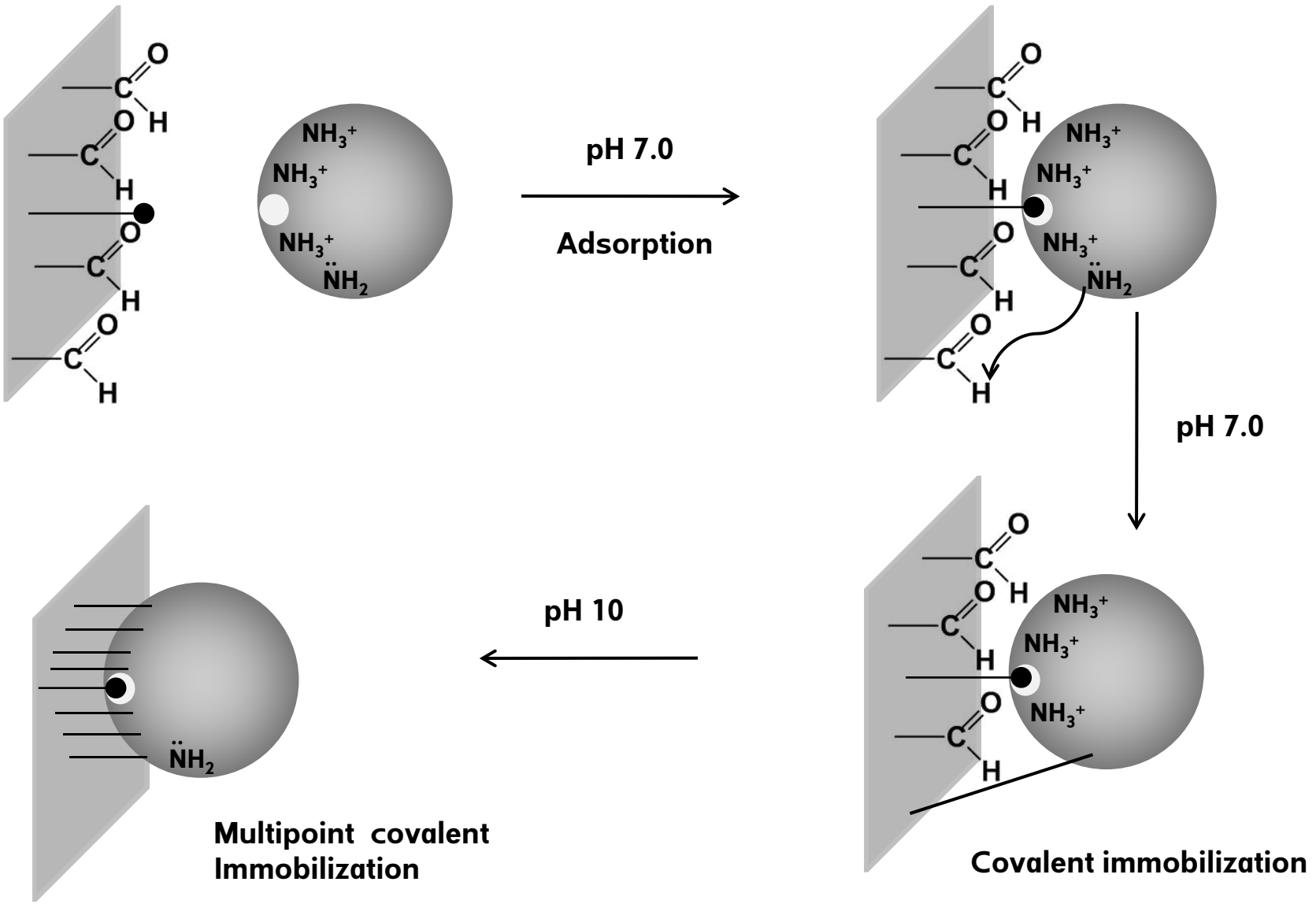


Unstable loop

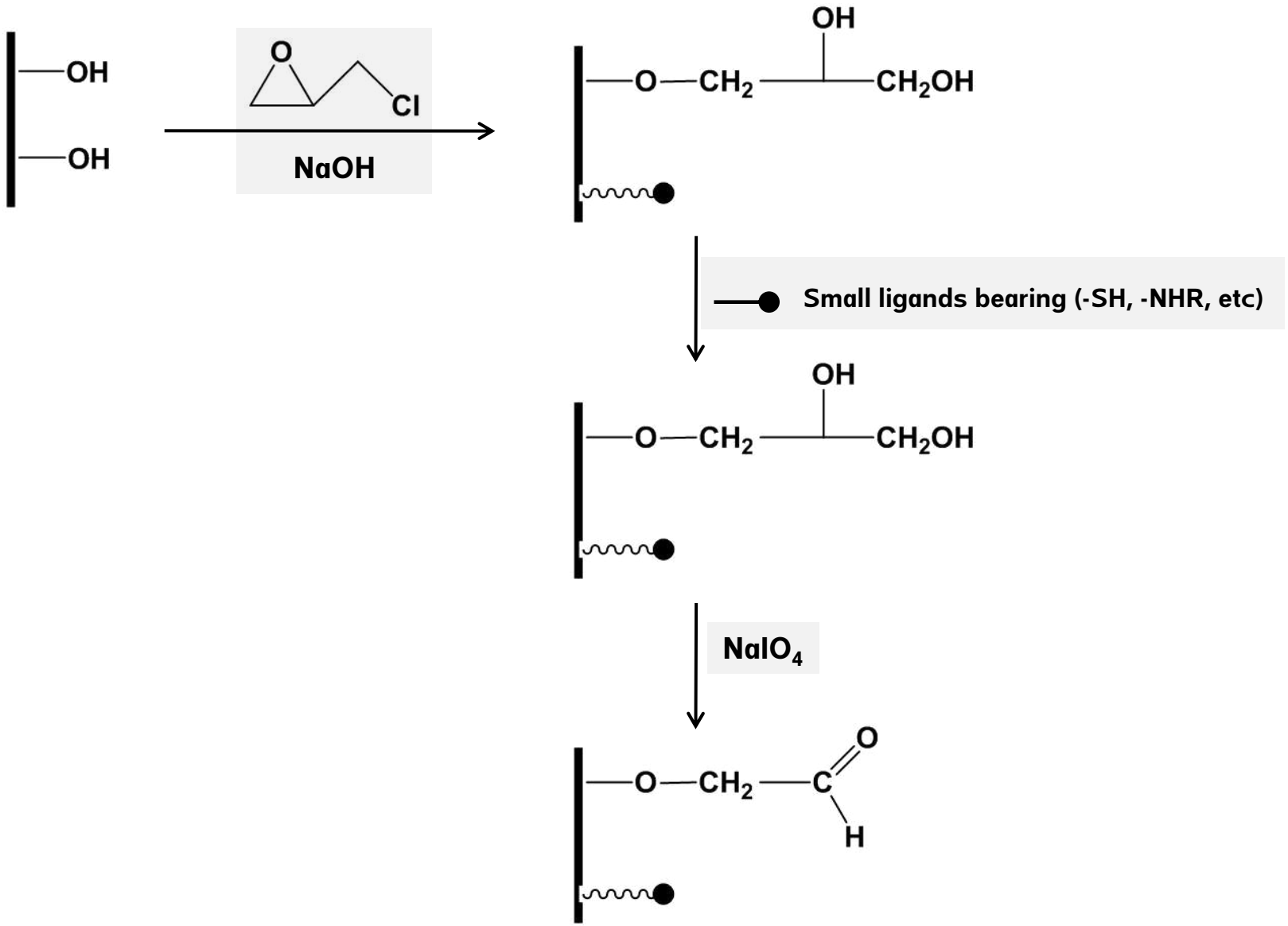


Close to active centre

Scheme 1

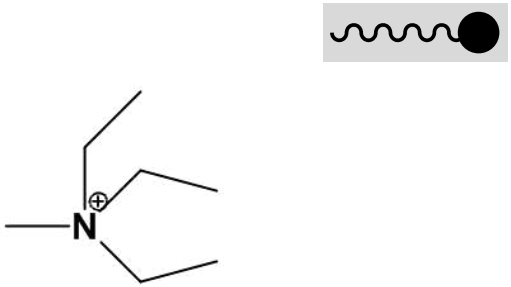

pH 7.0
Adsorption
pH 7.0
pH 10
Multipoint covalent
Immobilization
Covalent immobilization

Scheme 2


OH
OH
O
Cl
NaOH
OH
O
CH2
CH2OH
Small ligands bearing (-SH, -NHR, etc)
OH
O
CH2
CH2OH
NaIO4
O
O
CH2
C
H

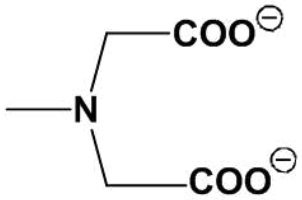
Scheme 3

Different ligands capable to adsorb proteins used in this manuscript

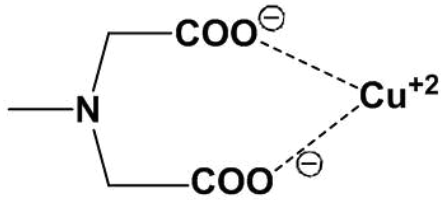


Ionized amino groups

Phenylboronic groups



Ionized carboxyl groups



Metal Chelates

Scheme 4

Figure 1

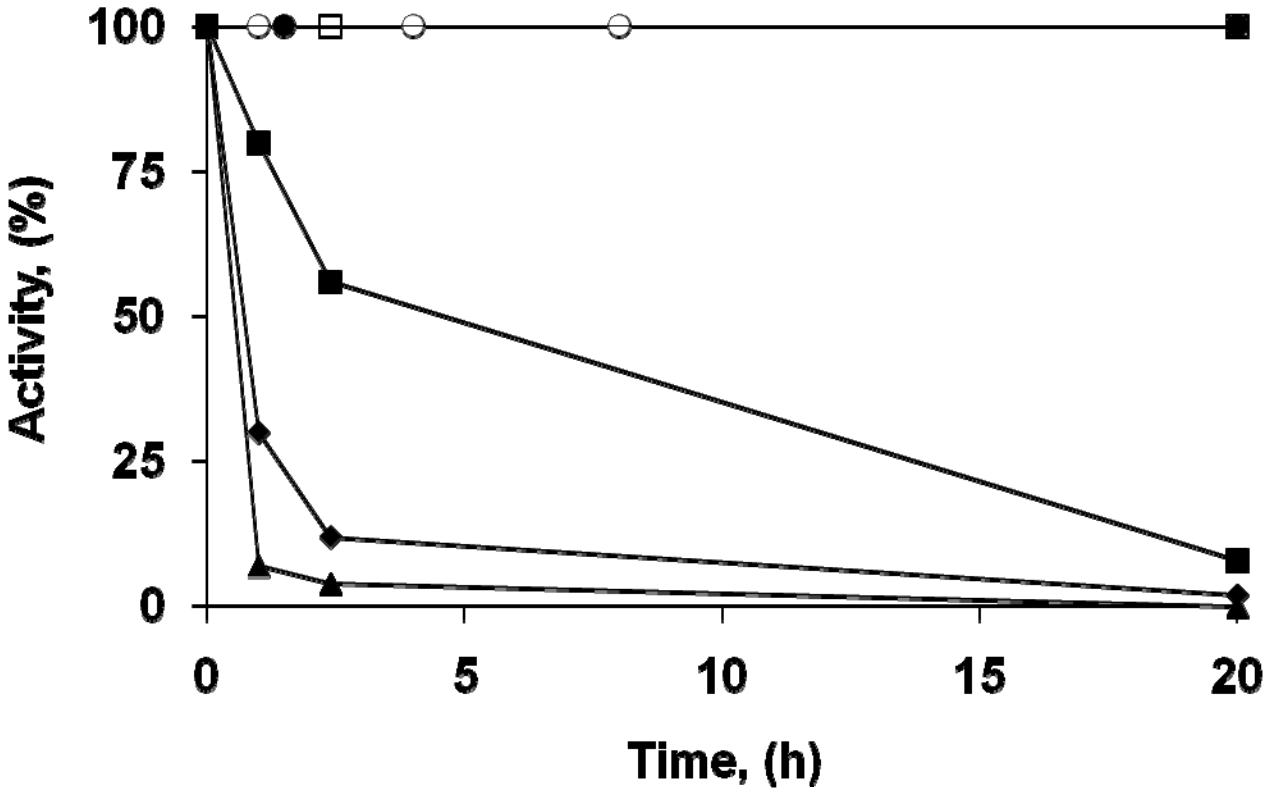


Figure 2

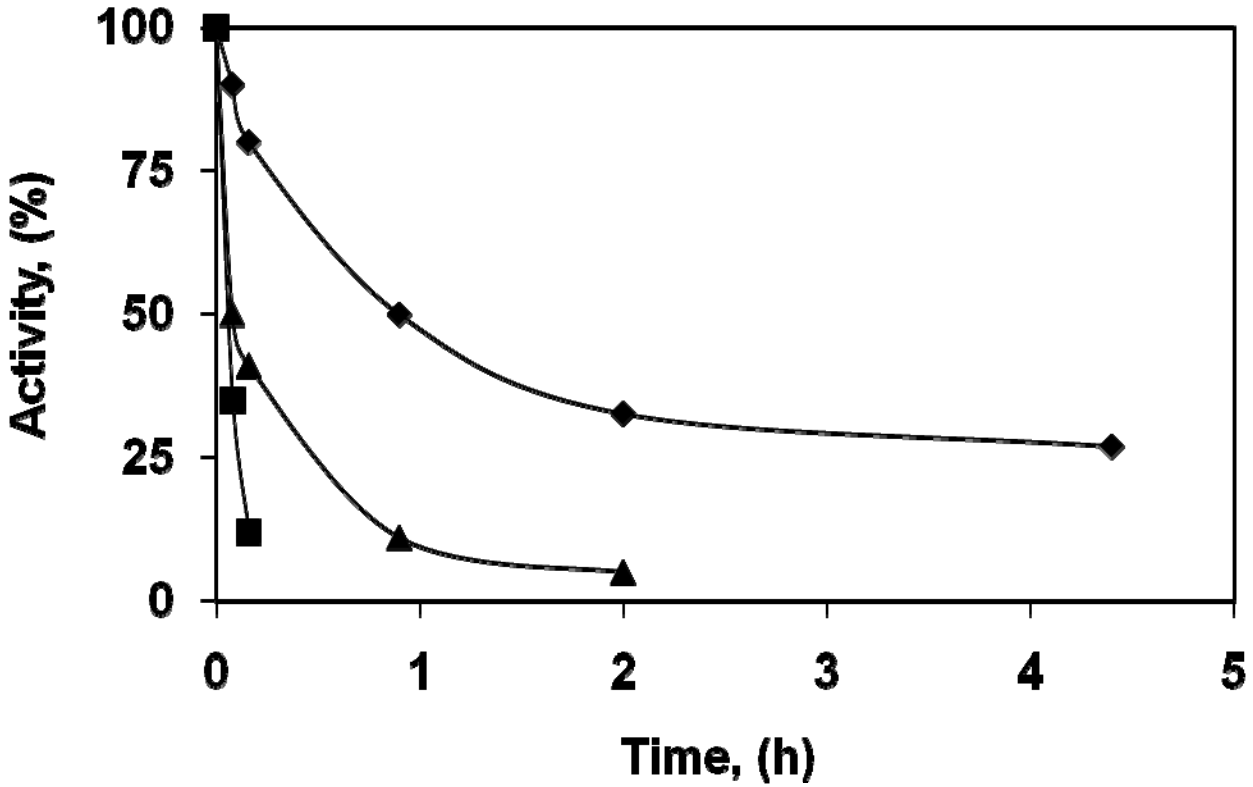


Figure 3 A

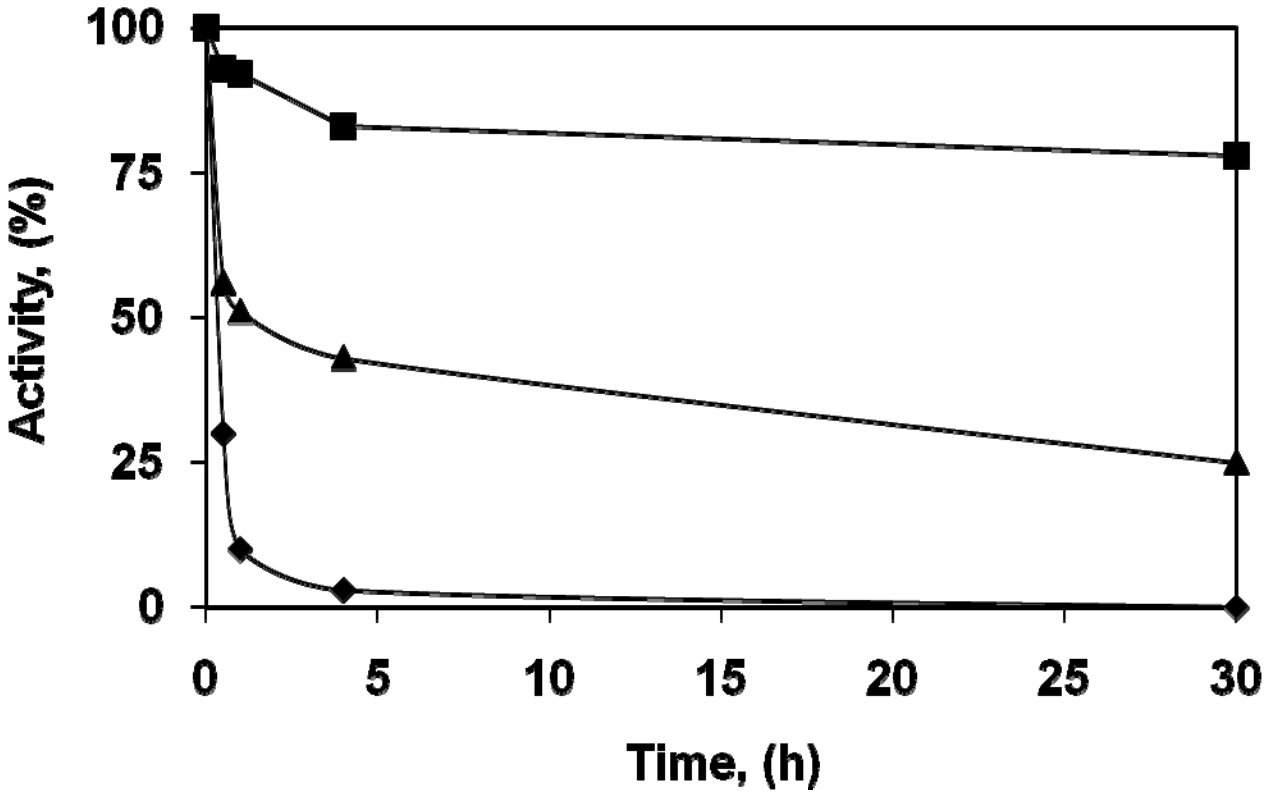


Figure 3 B

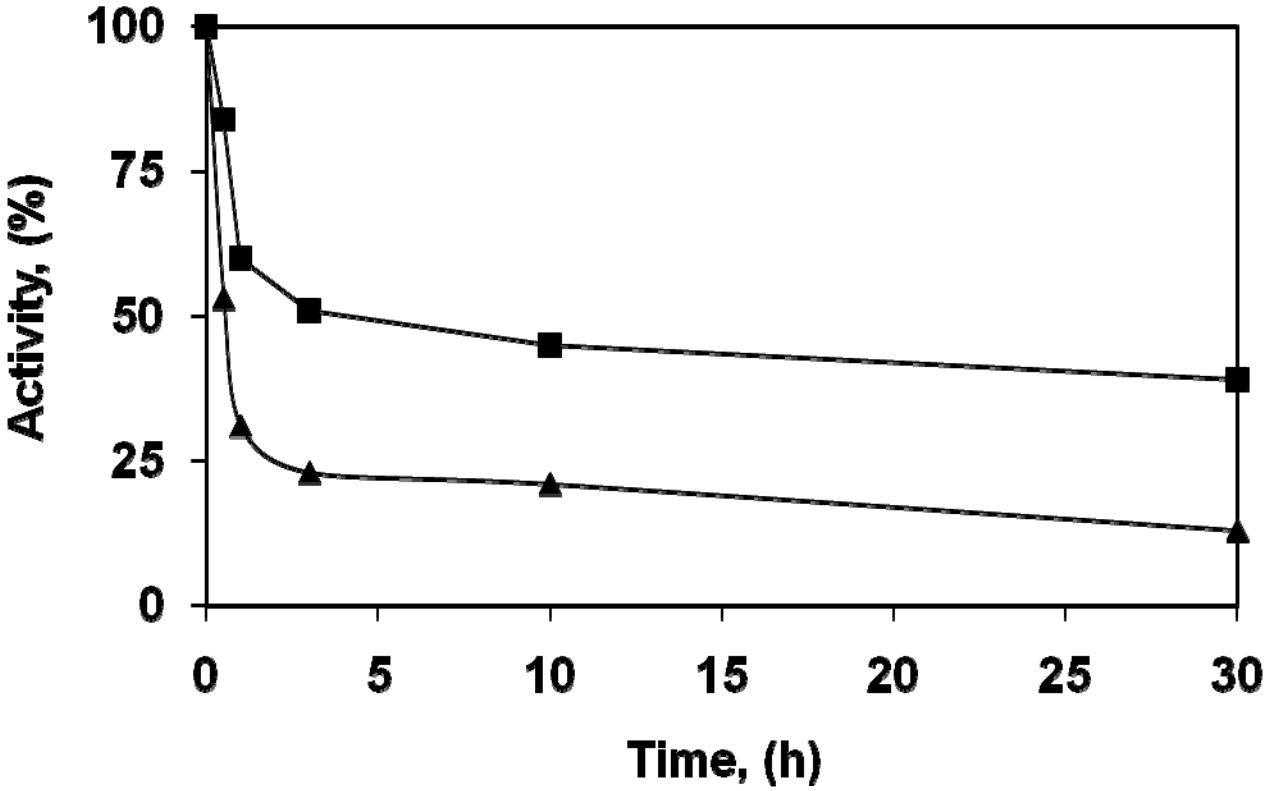


Figure 4

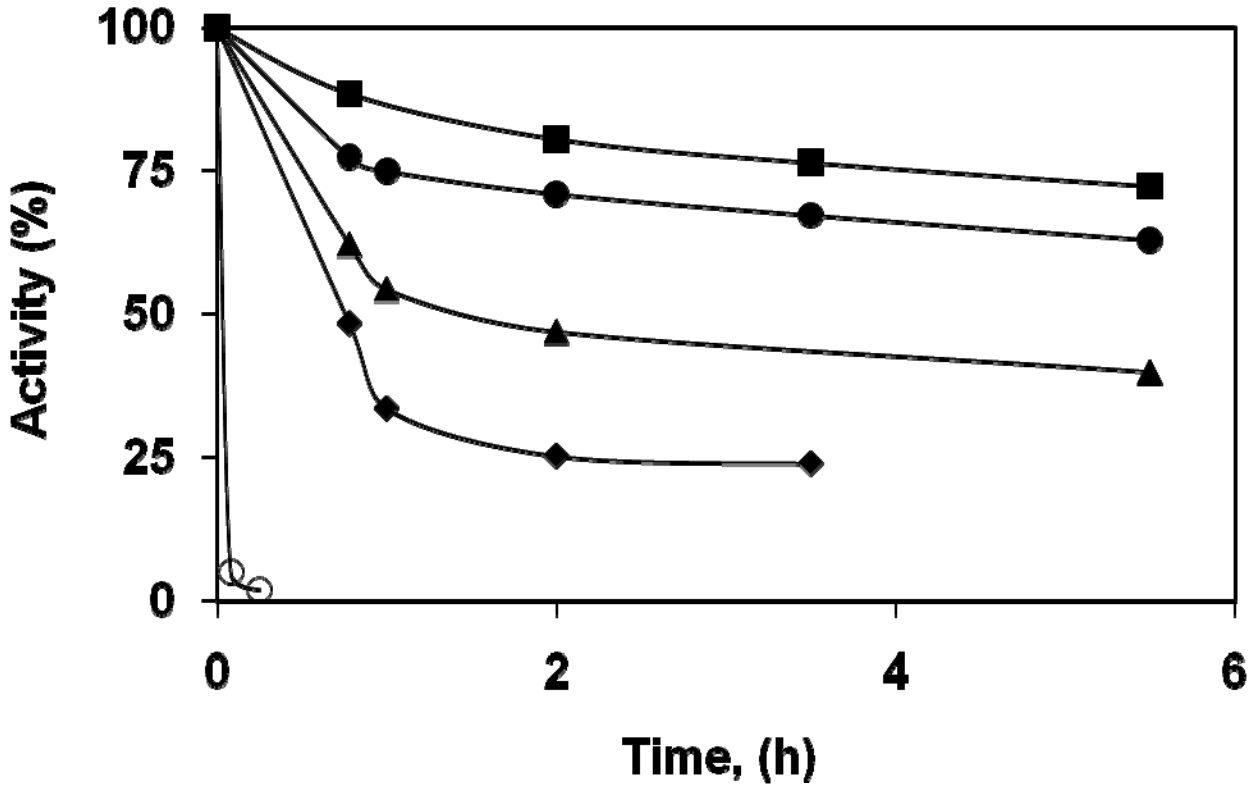


Table 1: Quantification of the reactive groups

Agarose	Adsorbent groups μmol/mL	Glyoxyl groups μmol/mL
10BCL	70±3	105±5
6BCL	30±1.5	45±2
4BCL	16±1	24±1.5

Table 2: Immobilization of BTL on amine-glyoxil supports

	30 min, pH 7.0	2 h, pH 7.0	12 h, pH 8.0
Immobilized enzyme (%)	80	100	100
Desorbed enzyme after incubation in 1 M NaCl (%)	80	50	10

Table 3: Immobilization of BTL on different glyoxyl supports

Support	Immobilized BTL (%)	Activity (%)	Activity after incubation at pH 10 (%)
Cu-CHO	94	21	11
Amino-CHO	100	90	90
Monofunctional CHO	100	60	60
Boronate-CHO	95	71	67

Table 4: Study of the enantioselectivity of different BTL derivatives. The study was performed using mandelic butyrate as described in methods.

Immobilized preparation	Activity (UI/min)	Asymmetry
Amino-glyoxyl- BTL	0.0017	8
Monofunctional glyoxyl -BTL	0.00075	2

Table 5: Different glyoxyl supports

support	pH of immobilization	Possible orientation of the enzyme
Monofunctional glyoxyl	10.0	Richest region on lysine
Monofunctional glyoxyl + thiols	7.0-8.0	Region with the most reactive amine group
Amine glyoxyl	7.0	Region with richest net negative charge
Chelate glyoxyl	7.0	Region richest in hystidine
Carboxylic glyoxyl	7.0	Region with richest net positive charge
Boronate glyoxyl	7.0	Region with highest affinity for boronate