

Effect of inactivation and reactivation conditions on activity recovery of enzyme catalysts

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Received April 16, 2013 / Invited Article
Published online: May 15, 2013
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Abstract

Enzymes are labile catalysts with reduced half-life time that can be however improved by immobilization and, furthermore, already inactivated catalyst can be recovered totally or partially, therefore allowing the large scale application of enzymes as process catalysts.

In recent years a few studies about reactivation of enzyme catalysts have been published as a strategy to prolong the catalyst lifetime. Reported results are very good, making this strategy an interesting tool to be applied to industrial process. These studies have been focused in the evaluation of different variables that may have a positive impact both in the rate and level of activity recovery, being then critical variables for conducting the reactivation process at productive scale.

The present work summarizes the studies done about reactivation strategies considering different variables: type of immobilization, enzyme-support interaction, level of catalyst inactivation prior to reactivation, temperature and presence of modulators.

Keywords: CLEAs, enzyme immobilization, enzyme reactivation, glyoxyl agarose, inactivation, organic cosolvents, unfolding - refolding.

INTRODUCTION

Enzymes have been used by decades as catalysts in the production and modification of foods and pharmaceuticals. Their potential applications will expand as long as the efficiency of catalyst use is increased. Enzyme immobilization not only allows catalyst reuse, but increases enzyme stability and allows its reactivation after partial exhaustion of its activity. Therefore, lifespan of the catalyst may increase many times as a consequence of enzyme immobilization.

Many strategies of immobilization with and without inert supports have been described and the catalysts characterized with respect to their potential applications as process catalysts. Among the many strategies proposed for covalent immobilization to inert supports, immobilization to glyoxyl agarose outstands in terms of the stabilization factors attainable.

Beyond the benefits associated with immobilization, reactivation after partial exhaustion of enzyme activity is another key issue associated with immobilization. The strategy of unfolding-refolding of proteins has been used for long as a technique for obtaining properly folded recombinant proteins. These proteins, when produced in microbial hosts, are usually unfolded and tend to form aggregates devoid of biological functionality. Only recently this idea of protein refolding has been applied as a tool for the reactivation of partially inactivated immobilized enzymes with the purpose of increasing their lifespan of use (Romero et al. 2009)

Catalyst reactivation will depend on many factors, being the strategy of immobilization a most important one. In the case of enzymes immobilized by adsorption, reactivation is not feasible, but it is in the case of enzyme immobilization by covalent linkage, being the matrix of immobilization an adequate scaffold for proper protein refolding and, as a consequence, for enzyme reactivation.

Activity level recovery by reactivation process will depend on many factors, which can be divided into those involved in the previous inactivation process and those involved in the refolding of the enzyme. These factors are listed in Figure 1.

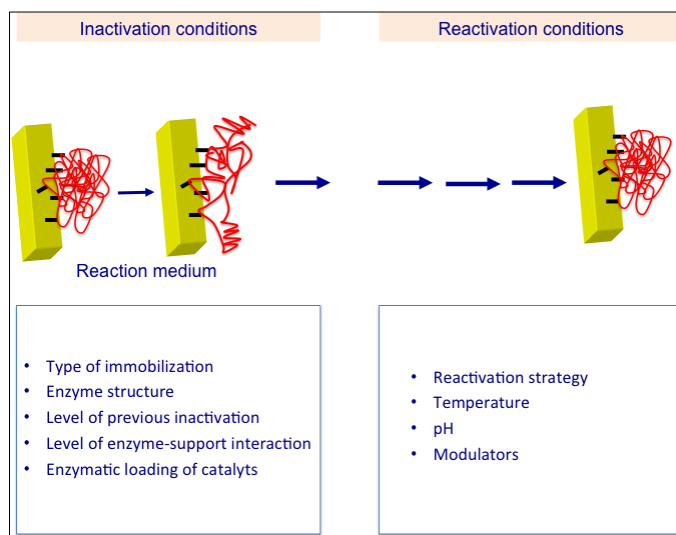


Fig. 1 Factors that affect the reactivation process.

ENZYME IMMOBILIZATION

Enzyme immobilization allows the reuse of the catalyst or its prolonged use in reactors operating in continuous mode (Sheldon, 2007; Brady and Jordaan, 2009; Wang et al. 2009). However, a few years ago, the potential of reactivation of partially inactivated immobilized enzymes provided an additional advantage to the use of these catalysts as process catalysts. There are a large number of methods of immobilization that can be broadly divided into those than involve the immobilization of the enzyme to solid matrices and those in which the enzyme is self immobilized in its own protein matrix without an inert support (CLEAs).

Covalent immobilization to solid supports is established between functional groups in the activated carrier and functional groups in the amino acid residues of the enzyme, like -OH, -SH, -NH₂, and -COOH. Covalent immobilization has been extensively studied and detailed information on methods and procedures is currently available (Cao, 2006; Guisán, 2006). Multi-point covalent attachment has allowed significant stabilization in a large number of enzymes. Immobilization on glyoxyl-agarose occurs through the enzyme surface region having the highest density of lysine residues and may be considered as the best protocol to obtain very intense enzyme-support multipoint covalent attachment (Mateo et. al. 2006; Pedroche et al. 2007; Bolívar et al. 2009a). Heterofunctional supports are those bearing more than one type of reactive groups able to establishing linkages with amino acid residues in the enzyme molecule, representing novel type catalysts derived from supports that have been traditionally used for enzyme immobilization (Mateo et al. 2007; Bolívar et al. 2009b; Bolívar et al. 2010; Mateo et al. 2010).

Non-covalent immobilization to solid supports implies different types of interactions between the enzyme and the support, including ionic bonds and hydrophobic interactions, but also weaker short-range interactions like van der Waals forces. They are simpler than covalent immobilization and many enzymes have been stabilized considering this immobilization strategy (Mateo et al. 2000; Pessela et

al. 2005; Torres et al. 2006; Fernández-Lorente et al. 2008; Brady and Jordaan, 2009; Palomo and Guisán, 2012). However, the reactivation of immobilized catalyst by adsorption is not considered, since the main cause of catalyst loss in this catalyst is due to desorption of the enzyme protein from the support.

Cross linked enzyme aggregates (CLEAs) are novel enzyme catalysts that combine the good properties of non-supported biocatalysts with simplicity and low cost of production, a distinctive advantage to cross-linked enzyme crystals (CLECs) that require of a highly purified enzyme which is not the case for CLEAs (Cao et al. 2000). Because of these advantages, CLEAs from many different enzymes have been produced and characterized (Gupta and Raghava, 2011; Sheldon, 2011). CLEAs are produced by cross-linking of protein aggregates produced by non-denaturing protein precipitation techniques. Despite being much cheaper and simple to prepare than CLECs, CLEAs have comparable specific activities and stabilities (Cao et al. 2000; Cao et al. 2003). However, no established protocols are available for the precipitation and crosslinking steps, so each enzyme represents a particular case that has to be studied and optimized (Roessl et al. 2010). CLEAs of penicillin acylase (Cao et al. 2000; Wilson et al. 2004; Pchelintsev et al. 2009) have been evaluated with respect to reactivation (Romero et al. 2009; Romero et al. 2012).

UNFOLDING-REFOLDING

Recombinant proteins over-expressed in microbial hosts are usually produced as insoluble particles known as inclusion bodies that usually lack biological functionality. To acquire functionality these inclusion bodies need to be subjected to a refolding process in which the protein reassumes its native three-dimensional structure (Nohara et al. 2000). Refolding is a key downstream operation in recombinant protein production at production scale in the pharmaceutical industry, but proper protein refolding is still a problem to solve despite its technological importance and the efforts done to solve it (Nohara et al. 2000). Recombinant proteins of therapeutic relevance already marketed are hormones (insulin and human growth hormone), blood coagulation factors (VIII, IX and VIIa) prescribed in the treatment of certain types of hemophilia, anticoagulants (tissue plasminogen activator, t-PA), hematopoietic factors (alpha and gamma interferons), monoclonal antibodies and so forth. Besides, a high number of recombinant proteins are now in different stages of approval by the regulatory agencies (Andersen and Krummen, 2002).

Protein refolding is a complex process conditioned by different properties of the protein, such as molecular size, number of sub-units, chain topology, oligomeric state and primary structure (Dobson and Karplus, 1999; Roman et al. 2010); refolding is also conditioned by the medium so additives like glycerol may be helpers of the reactivation process (Zhi et al. 1992; Mishra et al. 2007). Refolding is kinetically determined by the competence between the rates of refolding and molecular aggregation (Maachupalli-Reddy et al. 1997). Protein refolding can be represented by a first-order mechanism of reaction since it may be considered as a unimolecular process. However, the process of protein aggregation is of higher order since it is governed by intermolecular interactions (Maachupalli-Reddy et al. 1997; Agócs et al. 2010). Considering this, several studies have attempted to reduce the rate of reaction of protein aggregation, since it hampers the yield of refolding, which is a constraint for scale-up (Yazdanparast and Khodagholi, 2006). Such studies refer to the selection of adequate media for recombinant protein refolding as a downstream operation in its production process (Nohara et al. 2000). One of the strategies to reduce the impact of protein aggregation is to perform refolding at very low protein concentration, so that in the presence of a large volume of refolding buffer protein aggregation will be reduced, also diluting out the denaturing agent used for unfolding (Ikai et al. 1978; Kapoor et al. 1981; Yasuda et al. 1998; Katoh et al. 1999; Jaspard, 2000). Being protein concentration after refolding quite low, mixing problems may arise that will cause heterogeneity during refolding reducing process efficiency. A better strategy is the stepwise addition of the protein to the refolding buffer in a fed-batch operation, as applied to the production of recombinant lysozyme where a significant increase in the recovery of enzyme activity was obtained (Katoh et al. 1999). Other option is the use protein refolding helpers, like α -cyclodextrins, which prevent aggregation so that dilution of the protein solution can be considerably reduced. α -cyclodextrins not only reduce protein aggregation but also increase refolding rates (Karupiah and Sharma, 1995; Yazdanparast and Khodagholi, 2006; Kumar et al. 2009). Enzyme immobilization has been considered in a few cases as a tool for protein refolding. It is claimed that immobilization represents an adequate refolding system since intermolecular interactions, like aggregation, can be avoided and autolysis can also be prevented if present. Thermolysin has been immobilized with such purpose and a medium containing sodium and

potassium acetate was selected as refolding medium obtaining a 100% of recovery of enzyme activity (Nohara et al. 2000). Similar results in terms of yield were obtained with immobilized chymotrypsin and trypsin partly inactivated by incubation at high temperatures (Klibanov and Mozhaev, 1978; Martinek et al. 1980a; Martinek et al. 1980b; Mozhaev and Martinek, 1981; Mozhaev and Martinek, 1982; Mozhaev et al. 1987; Guisán et al. 1992a; Soler et al. 1997), derivatives of lipases (Guisán et al. 1996) and also with a recombinant enterokinase (Suh et al. 2005). In all these cases, immobilization favoured refolding and allowed in finding the most adequate media for refolding. In the case of partially inactivated glyoxyl-agarose immobilized chymotrypsin, the best conditions were an unfolding step by incubation at high concentrations of urea followed by refolding in aqueous buffer.

As shown above, most studies on protein refolding have been done in the context of recombinant protein production. However, despite its relevance for allowing enzymes to recover their native configuration, only recently this strategy has been considered from a process perspective as a way of recovering partially inactivated biocatalysts so expanding their life span of use (Figure 1).

ENZYME REACTIVATION

As biological catalysts, enzymes are inherently labile, which causes a gradual loss of activity, even in aqueous media and at rather mild conditions of pH and temperature (Illanes, 1999; Zaks, 2001; Illanes and Wilson, 2003; O'Fágáin, 2003). This problem is more serious in non-aqueous reaction media, where the lifespan of the biocatalyst is reduced to a point where the technological significance is lost (Gupta, 1992). Enzyme inactivation rates in organic cosolvents are usually high (Persichetti et al. 1995), even though there are some exceptions, like polyols and glymes (Illanes and Fajardo, 2001). Inactivation in organic cosolvents is caused by the conformational changes induced by the cosolvent on the enzyme structure as a consequence of water stripping in the enzyme microenvironment. In this way, active and stable biocatalysts are required to perform in such media (Koskinen and Klibanov, 1996; Klibanov, 1997; Ru et al. 1999).

The relationship between enzyme activity and enzyme hydration has been profusely reported (Rupley et al. 1983; Yamane et al. 1990; Khalaf et al. 1996). The expression of enzyme activity is conditioned by the water tightly bound to its structure. The presence of organic solvents may destroy the interaction between the tightly bound water molecules and the enzyme molecule so producing its inactivation (Zaks and Klibanov, 1988). The hydrophobicity of the organic solvent plays an important role in enzyme stability (Laane et al. 1985); when non-polar water-immiscible solvents are used enzymes will be more stable in the more hydrophobic solvents, since they will be kept out from the enzyme microenvironment to a higher extent. However, when polar water-miscible solvents (cosolvents) are used, the opposite effect will occur since in this case the comparatively more hydrophobic cosolvents will be most harmful to the enzyme structure by altering the hydrogen bonding and ionic interaction pattern between the enzyme and the medium. In principle, the mechanism of enzyme inactivation in cosolvent media is rather simple, because the chemical modification of the immobilized enzyme should be insignificant, since cosolvents are in most cases chemically inert. Immobilized enzymes are not inactivated by hydrophobic surfaces produced by air, immiscible solvents or drops of insoluble substrates, since these molecules cannot penetrate to the inner space of the porous matrix and also aggregation is impossible because of the total dispersion of the immobilized enzyme on the support (Fernández-Lorente et al. 2000). Under such conditions, the only cause of enzyme inactivation will be the conformational changes induced in the enzyme structure (Ross et al. 2000). It has been stated that enzyme inactivation will only reach the reversible phase of enzyme inactivation (Guisán et al. 1992b) so that, according to this, enzyme derivatives can recover some or all of its former activity by an adequate strategy of reactivation producing a proper refolding of the partially distorted enzyme configuration. In fact, it has been demonstrated that many proteins can spontaneously refold from a denatured state to its native conformation, after removing the condition that caused inactivation (Teipel and Koshland, 1971).

Studies on enzyme reactivation led to establish two possible alternatives for the recovery of enzyme activity (Figure 2): (a) reactivation by simple washing of the catalyst and resuspension in aqueous buffer; and (b) reactivation by unfolding-refolding, in which the enzyme is completely unfolded by the action of high concentration of a chaotropic agent (*i.e.* urea, guanidine) (Mozhaev et al. 1989) and then subjected to refolding in an aqueous environment (Soler et al. 1997).

The conditions affecting the reactivation process of a biocatalyst can be classified in those acting before (during inactivation) and those acting during reactivation. The former can be divided according to the immobilization procedure, protein structure, level of inactivation, inactivation, protein-protein crosslinking degree and protein loading in the catalyst. Among those acting during reactivation, the type of reactivation process, temperature, pH, presence of modulators and solvents can be mentioned. Factors that have been reported to affect the reactivation potential of immobilized enzymes are presented in the next section.

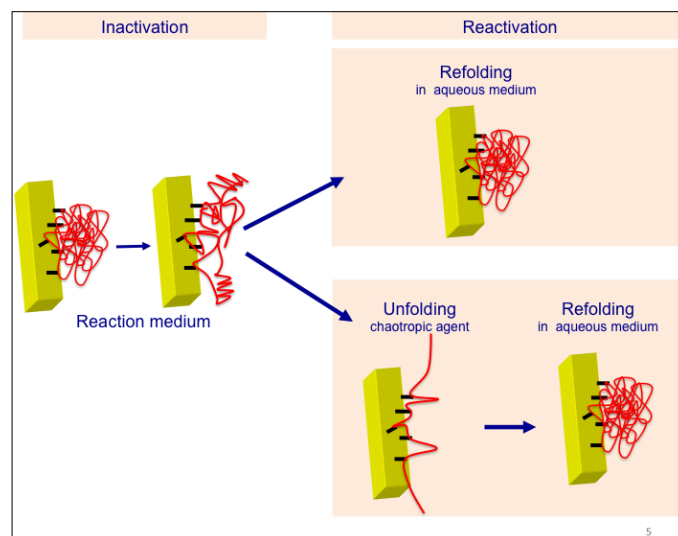


Fig. 2 Strategies of enzyme reactivation for the recovery of enzyme activity.

EFFECT OF CONDITIONS PREVIOUS TO REACTIVATION

Type of immobilization

Romero et al. (2009) compared two completely opposite immobilization strategies: one in which penicillin G acylase was immobilized without carrier (CLEA-PGA), and other where the enzyme was immobilized to glyoxyl-agarose by multi-point covalent attachment (GA-PGA). Both biocatalyst were inactivated and compared with respect to their reactivation capacity, quantified as the ratio of final recovered activity to initial (before inactivation) activity of the catalyst. Reactivation of the catalyst was done by incubation in aqueous medium, significant differences being observed between both catalysts. In the case of GA-PGA, total activity was recovered in the case of the catalyst previously inactivated to 75% of residual activity. However, at the same conditions, CLEA-PGA was not reactivated. Multipoint covalent attachment to a support is a distinctive advantage in terms of reactivation by conferring a scaffold which serves as a template for refolding (Soler et al. 1997; Mateo et al. 2006). In the case of CLEAs, due to the absence of a carrier, the proximity among enzyme molecules favours the interaction between them during inactivation and also during reactivation, which attempts to the possibility of achieving a correct tridimensional structure. High values of reactivation have been obtained also for a NADH oxidase enzyme variant from *Thermus thermophilus* HB27 immobilized on glyoxyl-agarose. The biocatalyst was inactivated in the presence of 60% (v/v) dioxane at pH 5 and 37°C, inactivation being complete after 20 hrs of incubation. The reactivation process, in this case, considered the strategy of incubation in aqueous media, 90% of initial activity being recovered. It is important to mention that the inactivation-reativation cycle for this enzyme was performed three times and similar results were obtained in every cycle (Rocha-Martín et al. 2011). The effect of amination of lipase from *Thermomyces lanuginosus* (TL) on their reactivation has been reported. Since support-protein interaction favours the reactivation of partially inactivated catalysts, amination contributed in this case by offering additional bonds for enzyme linkage to the support, since lipases are poor in surface lysine residues, that are the ones involved in the immobilization to glyoxyl-agarose support (Rodrigues et al. 2009a; Rodrigues et al. 2009b).

The effect of post-immobilization polymer addition on reactivation has been studied using the TL lipase. The previously aminated enzyme, was immobilized in glyoxyl-agarose, and after this, the biocatalyst was coated with hydrophilic and inert polymer (dextran) modified with glycine. The catalyst inactivated in 95% (v/v) dioxane was incubated in guanidine solution to favor the following step of refolding in aqueous media. After the whole inactivation-reativation process, the catalyst recovered 100% of its activity during three consecutive cycles. This study suggests that very hydrophilic and inert polymers coating the enzyme surface, help in the correct positioning of the hydrophilic and hydrophobic groups of the enzyme and, in this way, improve both the stability and the reactivation of the enzyme (Rodrigues et al. 2009a).

Godoy et al. (2011) studied the effect of the presence of reactive groups in the surface of the support on the reactivation of a lipase from *Geobacillus thermocatenulatus* (BTL2) immobilized in CNBr-agarose. The potentially reactive groups on the support surface after lipase immobilization were blocked with ethanolamine and ethylenediamine. The catalysts were inactivated in presence of guanidine and then reactivated in aqueous media at pH 9. Results showed that the catalyst blocked with ethanolamine exhibited 100% of initial activity, while the catalyst blocked with ethylenediamine recovered only 55% of initial activity. This result shows that reactive groups in the surface of the support promoted undesirable enzyme-support interactions during enzyme unfolding-refolding (Godoy et al. 2011).

Enzyme structure

The presence of cysteine residues on the enzyme surface allows oxidation processes during inactivation and reactivation of immobilized catalysts, reducing the recovery of enzyme activity. The cysteine residues may form disulfide bonds and also be oxidized to sulphinic and sulphonic acids, and even to sulfonyl amides formed between the oxidized thiol and the peptide bond (Harman et al. 1984). It is worthwhile mentioning that these effects are independent of the intensity of enzyme-support linkage (Godoy et al. 2011). Dithiothreitol (DTT) has been reported as very efficient reducing agent to prevent cysteine oxidation or disulfide bond formation (Karkhane et al. 2009). This effect was demonstrated with the lipase from *Geobacillus thermocatenulatus* immobilized both on CNBr-agarose as on glyoxyl-agarose. DTT was added both at the unfolding and refolding stages, attaining residual activities of 70% and 60% for glyoxyl-agarose-BTL2 and CNBr-agarosa-BTL2 respectively; these values duplicate those obtained for the corresponding catalysts without reducing agent addition (Godoy et al. 2011).

The effect of the presence of cysteine residues in the enzymatic structure on catalyst reactivation was evaluated with a lipase from *Geobacillus thermocatenulatus*. The study considered three mutants of BTL2: one BTL2 mutant with both native cysteine residues replaced by serine, and two mutants with one of the cysteine residues replaced by serine. All catalysts were immobilized in CNBr-agarose, and then inactivated in guanidine solution. During the reactivation process (without DTT), 94% of initial activity was obtained after reactivation with the mutant with both cysteines replaced, while 90% and 65% of initial activity was obtained with those with only one cysteine replacement (depending of cysteine position); with the catalyst prepared with the native enzyme only 30% of activity was recovered under same conditions. It is important to remark that this methodology can be applied to other enzymes containing free and reduced cysteines in their sequences (Godoy et al. 2011).

Reactivation studies on multimeric enzymes have also been reported. Bolívar et al. (2010) studied the reactivation of the trimeric glutamate dehydrogenase from *Thermus thermophilus* (GDH). The enzyme was immobilized in several supports to stabilize it and allow reactivation after unfolding with guanidine. Seven different strategies of immobilization were evaluated, achieving a 75% recovery of initial activity when the catalyst was immobilized in glyoxyl-agarose at pH 10. The multipoint attachment of the enzyme on this inert surface helped in the correct refolding of the enzyme, preventing unwanted interactions among close chains of different enzyme subunits. The reactivation levels achieved with the other GDH catalysts were low, which was related in some cases to the lost of enzyme subunits that were not covalently attached to the support, being leached out during the unfolding process with guanidine.

Level of previous inactivation

A recent report shows that, in general, reactivation is possible but the level attained after reactivation depends on the level of residual activity obtained after inactivation and prior to the reactivation step. As an example illustrating this, GA-PGA was inactivated in the presence of 70% (v/v) and 50% (v/v) dioxane, to obtain different residual activities: 75%, 50% and 25%. In all cases, activity of catalysts was recovered after reactivation; however, total recovery was only attained when inactivation was done to 75% residual activity, while 82% and 42% of activity was recovered when inactivation was done to 50% and 25% residual activity respectively. This shows that reactivation ability was dependent on the level of previous inactivation being less effective for catalysts that have lost a significant portion of their initial activity. In the same study, in the case of CLEA-PGA no clear effect of this variable was observed on catalyst reactivation in aqueous media (Romero et al. 2009).

Level of enzyme-support interaction

The effect of covalent interaction between enzyme protein and solid support was reported by Romero et al. (2012). In this case three different levels of protein-support interaction were evaluated with respect to reactivation ability. The enzyme PGA was immobilized in glyoxyl-agarose and the intensity of enzyme-support linkage in all catalysts was assessed in terms of their thermal stability, since every catalyst showed different half-life values according to the strength of linkage to the support. For example, the catalyst with less enzyme-support interaction was the most unstable and those prepared considering a higher intensity of interaction, were the ones with higher stability. Reactivation strategies were studied considering and not considering an unfolding step after inactivation. In the latter case, a higher reactivation of the catalyst was obtained at the stronger protein-support interaction, while those obtained at weaker protein-support interaction could not be reactivated. The tridimensional conformation of PGA certainly plays a role in this. This enzyme has two subunits, and when the catalyst is prepared with a low protein-support interaction level, it is probable that during the inactivation process some of the subunits are lost, which completely precludes catalyst reactivation. In the former case, this is, reactivation by the unfolding-refolding strategy, interesting results were obtained: the catalyst prepared with lower protein-support interaction was not reactivated suggesting that subunit dissociation of the few immobilized enzyme molecules retaining both subunits may have occurred during the unfolding stage; the catalyst with intermediate interaction level could be reactivated up to activity values similar to those catalysts with higher interaction levels, suggesting that in this case protein unfolding helped in removing unwanted interactions in the protein structure, allowing a proper refolding.

Protein loading of catalyst

The effect of enzyme protein loading of catalysts on reactivation has been studied with PGA-GA (Romero et al. 2009). In this work, six PGA-GA with protein loadings from 2.4 to 35.4 mg protein per gram of support were compared. Reactivation was done according to the unfolding-refolding strategy because drastic conditions during unfolding allow a better appraisal of possible interactions among neighbour protein molecules when the enzyme is subjected to an inactivation-reativation cycle. The results showed that full recovery of enzyme activity was obtained with catalyst protein loads up to 7.7 mg protein per gram of support. At higher protein loads, recovery was significantly impaired because of too intense protein-protein interaction during biocatalyst inactivation and reactivation. Besides, results suggest that enzyme molecules should be sufficiently spaced in the support to avoid protein-protein interaction during the inactivation and reactivation stages, which favours the use of catalysts with low protein loads. However, catalyst with low enzyme activity are not appropriate for production purposes, establishing a compromise between catalyst activity and reactivation potential, which is something important to bear in mind when optimizing enzymatic processes.

EFFECT OF REACTIVATION CONDITIONS

Temperature

Temperature effect on the reactivation process was studied with GA-PGA subjected to inactivation in the presence of 70% (v/v) dioxane down to a residual activity of 25%. Reactivation in aqueous medium was performed at different temperatures and time profiles of reactivation are presented in Figure 3.

It can be observed that temperature has a clear influence on catalyst reactivation rate but not in the final level of reactivation that is similar at all studied temperatures (at 5°C reactivation is so slow that the end point of reactivation exceeds the time studied). On the other hand, at the higher temperatures, a decrease in recovered activity is observed after several hours of incubation (15 and 10 hrs for 40°C and 50°C, respectively), which is the consequence of the thermal inactivation of the catalyst after prolonged incubation.

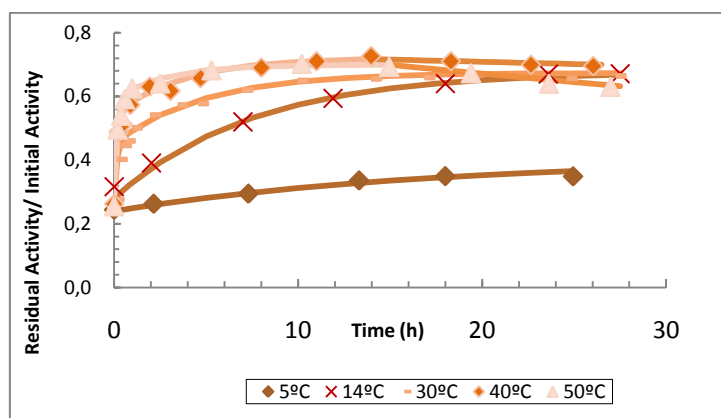


Fig. 3 Time course of GA-PGA reactivation at different temperatures.

pH

The reactivation of GA-PGA at different pHs (6.4, 7.4 and 8.4) was studied. That pH range was selected based on usual conditions reported for this enzyme (Guranda et al. 2004). Results show that pH exerts no influence on reactivation in the range studied final enzyme recovered activity being in all cases approximately 60% of initial.

A quite different result was obtained with glyoxyl-agarose-BTL2 and CNBr-agarose-BTL2. The evaluation was done in the 5 to 9 pH range, in the presence and absence of DTT during both inactivation and reactivation stages. Marked effects of pH were evidenced on the reactivation of both catalysts. CNBr-agarose-BTL2 recovered 100% of its initial activity when the unfolding and refolding stages were done at pH 9, while the best level of reactivation of glyoxyl-agarose-BTL2 was obtained at pH 5 (Godoy et al. 2011).

For reactivation of trimeric glutamate dehydrogenase from *Thermus thermophilus* (GDH), immobilized in glyoxyl-agarose support, two values of pH (5 and 9), a 50°C were evaluated, and with this conditions was possible to attain a total activity recovery for the catalyst previously inactivated with guanidine at pH 9 (Bolívar et al. 2010).

Modulators

Miranda et al. (2011) studied the effect of addition of ethyleneglycol, polyethyleneglycol (PEG) and glycerol in the reactivation media of GA-PGA. The study was carried out with catalysts previously inactivated in the presence of 70% (v/v) dioxane, down to 50% of initial activity. The effect of addition of cosolvents in the 0-100% (v/v) range was also studied. Result showed that up to 30% (v/v) cosolvent, reactivation was favoured in terms of rate and final level of reactivation. At concentration higher than 50% (v/v) cosolvent no improvement was obtained, and even a negative effect during reactivation process was observed. Best results were obtained with ethyleneglycol and PEG at 30% (v/v). An increase in recovery of enzyme activity from 36.0 to 62.8% (with respect to fully aqueous medium) was obtained using the unfolding-refolding strategy for catalyst reactivation.

In the case of catalytic modulators, competitive inhibitors (phenylacetic acid and 2-thienylacetic acid) were added to the reactivation media, causing reduction in the recovery of enzyme activity, while non-

competitive inhibitors (7-amino 3-desacetoxycephalosporanic acid, 7-ADCA, and 6-aminopenicillanic acid, 6-APA) addition caused an increase of this parameter. Combining cosolvent and catalytic modulators, best results in both reactivation strategies were obtained with 30% (v/v) ethyleneglycol plus 100 mM 7-ADCA, with 99% recovery of enzyme activity. Reactivation rates were higher in the case of direct incubation (not considering unfolding) in reactivation media, indicating that this is the best strategy for PGA reactivation when cosolvents and modulators are included in the reactivation media.

Godoy et al (2011) studied the effect of several modulators during the reactivation process for lipase BTL2 immobilized in glyoxyl-agarose. In all cases, the enzyme was inactivated in the presence of guanidine. The modulators studied were: Triton X-100, TMAO, glycerol, PEG 1500, dextran 1500 and trehalose, at different concentrations. Best results were obtained with PEG 1500 and dextran 1500, with 97% and 99% recovered activity respectively obtained at high reactivation rates.

In the case of lipases, a powerful tool for reactivation is the use of detergents. It has been shown that the interaction of the hydrophobic zone of the detergent with the hydrophobic zone in the active site of the lipase helps by stabilizing its open (active) configuration and promoting the proper refolding of the enzyme protein. One example of this effect is the reactivation of a lipase from *Thermomyces lanuginosus* (TLL-A), immobilized in glyoxyl-agarose support and coated with dextran modified with glycine. A significant reactivation of this catalyst after thermal inactivation was not possible in the absence of detergents. However, when enzyme activity was measured in the presence of detergent (CTAB) the catalyst exhibited 100% of initial activity (Rodrigues et al. 2009a).

CONCLUDING REMARKS

Preliminary studies on enzyme reactivation were published before the subject of protein refolding acquired technological importance with respect to recombinant protein production. The idea of protein refolding has been, however, enlightening with respect to enzyme reactivation after activity exhaustion. Enzyme reactivation requires a thorough study to identify the critical variables affecting it and determine their optimal values with respect to the level and rate of reactivation. This has to be done case by case, since information is for the moment scarce and no general guidelines exist with respect to enzyme reactivation. Being an important issue with respect to catalyst efficiency of use and having this in many cases a strong impact on production costs, the potential of catalyst reactivation should be considered as a part of the information required to make a global analysis of the process, including enzyme immobilization, enzyme inactivation under operating conditions and catalyst reuse. In fact, enzyme reactivation is clearly dependent on the previous history of the catalyst from its production to its utilization stage. The concept of catalyst memory is to be considered when studying reactivation.

Immobilization, by providing a scaffold for enzyme molecules, represent a framework to orient the enzyme structure towards an active configuration during refolding; this is not attainable in enzymes dissolved in the reaction medium and will have a lesser effect on carrier-free than in carrier-bound immobilized enzymes, as we have observed working with glyoxyl agarose bound and CLEAs of penicillin G acylase.

Immobilized enzymes can certainly be reactivated to a considerable extent, especially when previous inactivation has not gone too far down to the point of irreversible inactivation. This is particularly important in the case of enzymes inactivated by non-aqueous media where almost complete recovery can be attained under appropriate conditions; this in turn has a profound technological meaning in the context of enzyme catalysis in organic synthesis where non-aqueous media are often required. Enzyme reactivation after thermal inactivation is a more serious challenge because irreversible inactivation is likely to be preponderant in this case.

Even though there is much information available on enzyme reactivation, most studies lack a process perspective, so future studies should pinpoint to the technological meaning of including reactivation stages during the span of life of enzyme catalysts during reactor operation. Good news is that considerable reactivation after enzyme activity exhaustion is attainable. Challenge is to prove that the benefit of including this new stage during reactor operation is meaningful from the perspective of process cost. Information will be gathered in the forthcoming years to feed this type of analysis.

Financial support: This work was funded by Fondecyt-Chile Grant Nr 1100323.

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How to reference this article:

WILSON, L.; ILLANES, A. and ROMERO, O. (2013). Effect of inactivation and reactivation conditions on activity recovery of enzyme catalysts. *Electronic Journal of Biotechnology*, vol. 16, no. 3. <http://dx.doi.org/10.2225/vol16-issue3-fulltext-11>