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Complete reactivation of immobilized derivatives of a trimeric glutamate dehydrogenase from *Thermus thermophillus*

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ABSTRACT

First, the enzyme immobilized on cyanide bromide agarose beads (CNBr) (that did not involve all enzyme subunits in the immobilization) has been crosslinked with aldehyde-dextran. This preparation did not any longer release enzyme subunits and become fully stable at pH 4 and 25 °C.

Then, the stabilities of many different enzyme preparations (enzyme immobilized on CNBr, that derivative further crosslinked with aldehyde-dextran, enzyme immobilized on highly activated amino-epoxy supports, GDH immobilized on supports having a few animo groups and many epoxy groups, GDH immobilized on glyoxyl-agarose beads at pH 7, and that preparation further incubated at pH 10, and finally the enzyme immobilized on this support directly at pH 10) were compared at pH 4 and high temperatures, conditions where both dissociation and distortion play a relevant role in the enzyme inactivation. The most stable preparation was that prepared at pH 7 and incubated at pH 10, followed by GDH immobilized on amino and epoxy supports and the third one was the enzyme immobilized on glyoxyl-agarose at pH 10.

The incubation of all enzyme preparations in saturated guanidine solutions produced the full inactivation of all enzyme preparations. When not all enzyme subunits were immobilized, activity was not recovered at all. Among the other derivatives, only glyoxyl preparations (the most inert supports and those where a more intense multipoint covalent attachment were expected) gave significant reactivation when re-incubated in aqueous medium. After optimization of the reactivation conditions, the enzyme immobilized at pH 7 and later incubated at pH 10 recovered 100% of the enzyme activity.

1. Introduction

The relatively low enzyme stability under industrially relevant conditions is one of the problems that are hindering the implementation of these interesting biocatalysts [1–4].

In an industrial reactor, enzymes are usually employed in an immobilized form [5–7]. In this sense, the reactivation of these enzymes (after their partial inactivation) in these industrial reactors would increase the operational half-life of the biocatalyst [8]. At first glance, the reactivation of immobilized enzymes may have some advantages compared to the reactivation of free enzymes. For example, immobilized enzymes cannot aggregate during any step of inactivation or reactivation [9]. In fact, in many instances enzymes having a poly-His tag and that are produced as inclusion bodies, are unfolded and refolded after their immobilization on IMAC columns to get an active molecule [10–12].

Moreover, if an intense multipoint covalent attachment of the enzyme and the support has been achieved, not only is the enzyme more stable [1,13], but also these points, fixed to a rigid support, act as reference points that help to reach higher activity recoveries of the enzyme [8,14–17].

Multimeric enzymes are an especially complex case. These enzymes are produced "*in vivo*" as individual monomers that later assemble by a set of multipoint non-covalent and weak interactions (that globally are strong enough to keep the multimer assembled), the dissociation of subunits being in many instances the main reason for enzyme inactivation [18,19]. Reactivation of these enzymes needs not only to get the correct folding of each individual monomer, but also the correct assembly of the multimer. There are many examples of unfolding–refolding of multimeric soluble enzymes, in many cases with good results [20–25]. However, there are no reports on the reactivation of immobilized multimeric enzymes. Penicillin G acylase and chymotrypsin are two enzymes that have been reactivated in soluble [26,27] and immobilized [15–17] form, but these enzymes are produced as a monomer and are processed later, being not "real" multimeric enzymes [28–32].

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In the case of multimeric enzymes, in order to reactivate them some aspects should be considered:

1. If some subunits are not attached to the support, they can be lost in the washing steps of the reactor.
2. The proximity of one subunit to the other may produce incorrect structures by interaction between chains belonging to different enzyme subunits.

The first problem may be solved if the immobilization of the enzyme involves all enzyme subunits [13]. If that is not the case, further crosslinking with poly-functional polymers, like aldehyde-dextran [33,34], may be a good option. After reduction, these polymers are very inert and should not produce undesired enzyme–polymer interactions.

The solution to the second problem may be more complex. The use of enzymes immobilized via multiple points to the support may reduce the mobility of the protein chains, and that can make the formation of subunit–subunit interactions more difficult.

Glutamate dehydrogenase (GDH) is an enzyme that may be used in the regeneration of cofactors $NAD(P)^+$ or NAD(P)H coupled to other dehydrogenases as biocatalyst [35–38] or as biosensor of different compounds [39–41]. The GDH from *Thermus thermophilus* is a trimeric enzyme with good thermostability, which has been recently cloned in *Escherichia coli* [42]. In this manuscript, we intend to reactivate several immobilized preparations of the enzyme glutamate dehydrogenase from *T. thermophilus*. The enzyme is very stable at neutral or basic pH values. However, at acidic pH values the enzyme stability is very low due to subunit dissociation [42]. This problem was solved by multisubunit immobilization [42].

Here, we have compared the stabilities and the reactivation possibilities of some immobilized preparations of GDH that present very different situations:

- GDH immobilized for short times at pH 7 on cyanogen bromide agarose beads (CNBr-agarose), that did not immobilize all the enzyme subunits [42].
- A new GDH preparation from the previous derivative, that will be treated with aldehyde-dextran to crosslink the enzyme subunits and prevent enzyme dissociation [33,34].
- GDH immobilized on glyoxyl-agarose at pH 10. This preparation had all the enzyme subunits immobilized on the support, by the face of the trimer having the highest amount of Lys groups, and stabilized by multipoint covalent attachment [42].
- GDH immobilized on glyoxyl-agarose beads at pH 7. Although glyoxyl-agarose cannot immobilize most enzymes at pH 7 because it requires a first multipoint covalent immobilization [43,44], some multimeric enzymes are an exception. This is because they have several reactive amino groups at pH 7 [45]. This preparation had all the enzyme subunits immobilized on the support, but a very limited multipoint covalent attachment of each subunit [46].
- GDH immobilized on glyoxyl-agarose at pH 7 and later incubated at pH 10, to favour the enzyme–support reaction [46].
- GDH immobilized on a support highly activated with amino-epoxy groups [47]. This support did not immobilize all the enzyme subunits [48].
- GDH immobilized on supports activated with many epoxy groups and just a few amino groups [48,49]. The first immobilization is via a multi-ionic exchange adsorption of the enzyme on the support involving the 3 subunits, the incubation at alkaline pH value permitted the multipoint immobilization of all enzyme subunits [48].

2. Materials and methods

2.1. Materials

Nicotinamide adenine dinucleotide (NAD^+) was purchased from Jülich Fine Chemicals (Jülich, Germany). Cyanogen bromide sepharose 4B and crosslinked agarose beads (6%) were from Amersham Biosciences (Uppsala, Sweden). Glutamic acid and alpha-keto glutaric acid were supplied by Sigma–Aldrich Chem. Co (St. Louis, USA). Glyoxyl-agarose beads were prepared as previously described [50]. All other reagents were of analytical grade. Glutamate dehydrogenase from *T. thermophilus* was over expressed in *E. coli* and purified as published elsewhere [42]. Protein concentration was determined using Bradford's method [51]. Amino supports (monoaminoethyl-N-ethyl (MANAE)-agarose) with different degrees of activation were prepared as previously described [52]. Aldehyde-dextran (40 kDa) was prepared as previously reported [53].

2.2. Enzyme assays

The activities of the different GDH preparations were analyzed by the increase in absorbance at 340 nm corresponding to the formation of NADH concomitant to glutamic acid oxidation. A sample of enzymatic preparation (25–400 μL) was added to a cell with 2 mL of 250 mM glutamic acid and 100 μL of 100 mM NAD^+ in 100 mM sodium phosphate at pH 8.0 and 66 °C. Activity was determined from the observed slope from second 20 to second 100. One GDH unit (U) was defined as the amount of enzyme required to oxidize 1 μmol of glutamic acid per minute at pH 8 and 66 °C. The glutamate dehydrogenase preparation used for immobilization assays had a specific activity of 2.45 U/mg of protein and 1.5 U/mL.

2.3. Preparation of the supports

2.3.1. Preparation of heterofunctional amino and epoxy agarose beads

Controlled amination of the support was performed as previously described [54]. 10 wet g of epoxy agarose were incubated in 60 mL of 2% (v/v) ethylenediamine at pH 8.5 for different times (from 15 min to 24 h) under very gentle stirring. Then, the supports were washed with distilled water, 1 M NaCl and finally with distilled water. The degree of modification was quantified by titration of the amino groups introduced in the support as it was previously described [55,56]: 1 mL of sodium bicarbonate 100 mM pH 8,5 and 500 μL of TNBS (5%) were added to 1 mL of activated support. The suspension was gently stirred at 25 °C for 30 min and then the support was washed with sodium phosphate at pH 7. Finally, the absorbance of the support suspended in sodium phosphate was read at 420 nm. The reagent blank consists in similar amount of the support without amino groups.

2.3.2. Preparation of amino-epoxy agarose beads

The support was prepared as previously described with minor modifications [47]. 15 mL of MANAE support activated with 40 μmol/g of support [52] were suspended in 160 mL of 100 mM sodium bicarbonate at pH 9. Then 24 mL of acetone and 40 mL of 1,4-butanediol diglycidyl ether were added. The final pH value of the mixture was adjusted to 9. The suspension was incubated for 12 h, and after this period, it was washed with an excess of distilled water. Epoxy content was determined by chemical modification with iminodiacetic acid (IDA) and further adsorption of copper as previously described [57]. Firstly, the epoxy groups of the supports were substituted with IDA by the following protocol: 5 g of each support were incubated with 5.5 mL of 2 M IDA dissolved in 0.1 M sodium bicarbonate pH 11, for 16 h at 25 °C. Then, 2 g of each support were incubated with 0.17 M copper sulfate for 2 h at room temperature. After thoroughly washing with deionized water, the cooper adsorbed on to iminodiacetic moieties was desorbed with 0.1 M EDTA in 0.1 M sodium phosphate pH 7.0. The colour of the desorbed cooper solution was measured at 730 nm. Agarose without activation was used as blank sample.

2.4. Preparation of the different immobilized GDH preparations

An enzyme solution at the indicated pH and conditions was mixed with the specified amount of different supports. At different times, samples of the supernatant, the support-enzyme suspension, and an enzyme solution incubated in the presence of the inert support were taken, and the activity and/or the protein concentration was assayed. All the experiments were performed using less than 4 U/mL of support in order to avoid diffusion problems that could alter the apparent enzyme stability. The recovered activities are collected in Table 1.

2.4.1. Immobilization on CNBr-activated sepharose 4B

The immobilization was carried out by adding 2 g of the CNBr-activated support to 18 mL of 100 mM sodium phosphate at pH 7 containing 8 U of GDH. The suspension was kept under mild stirring for different times at 4 °C for 15 min [42]. Afterwards, the support was filtered and washed with 100 mM sodium bicarbonate at pH 8 and 4 °C, and incubated for 2 h in 1 M ethanolamine at pH 8 at 4 °C to block the remaining reactive groups on the support surface. Finally, the immobilized preparation was washed with distilled water.

Treatment of CNBr-GDH with aldehyde-dextran: This was performed as previously described [33,34]. Fully oxidized dextran was used for this modification. 5 g of