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Purification and stabilization of a glutamate dehygrogenase from *Thermus thermophilus* via oriented multisubunit plus multipoint covalent immobilization

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ABSTRACT

The immobilization of a glutamate dehydrogenase from *Thermus thermophilus* (GDH) on glyoxyl agarose beads at pH 7 has permitted to perform the immobilization, purification and stabilization of this interesting enzyme. It was cloned in *Escherichia coli* and a first thermal shock of the crude preparation destroyed most mesophilic multimeric proteins. Glyoxyl agarose can only immobilize enzymes via a multipoint and simultaneous attachment. Therefore, only proteins having several terminal amino groups in a position that permits their interaction with a flat surface can be immobilized. GDH became rapidly immobilized at pH 7 and its multimeric structure became stabilized as evidenced by SDS-PAGE. This derivative was stable at acidic pH value while the non-stabilized enzyme was very unstable under these conditions due to subunit dissociation. After immobilization, a further incubation at pH 10 improved enzyme stability under any inactivating conditions by increasing the enzyme–support bonds. In fact, GDH immobilized at pH 7 and incubated at pH 10 preserved more activity than GDH directly immobilized at pH 10 (50% versus 15% after 24 h of incubation) and was also more stable (1.5- to 3-fold, depending on the conditions).

This method could be extended to any other multimeric enzyme expressed in mesophilic hosts.

1. Introduction

Enzyme immobilization permits to obtain a heterogeneous catalyst, and if properly designed, improved enzyme stability may be achieved [1–4]. On the other hand, there are many protocols to purify proteins, as for example chromatographic protocols [5–7]. In this sense, the most interesting chromatographic methods of enzyme purification at industrial level will be those involving the selective adsorption of the target protein [8,9].

Obviously, if the immobilization step could be coupled to the purification of the target protein, it may be of great interest, saving time and costs in the development of the biocatalyst. There are some strategies that permit to couple these steps in the development of an immobilized enzyme preparation: use of immobilized antibodies [10–12], adsorption of lipases on hydrophobic supports [13,14], use of heterofunctional supports having an affinity moiety and groups able to give covalent attachment [15,16], or using restrictive conditions during immobilization [17,18]. However, a one-step purification—immobilization would have a special interest if the immobilization step may be used to increase the stability of the biocatalysts.

Multipoint covalent attachment of enzymes on activated supports is a very powerful tool to stabilize proteins: all the groups involved in the immobilization should keep their relative positions unchanged during any conformational change induced by any distorting agent [1–3]. Glyoxyl agarose is an activated support that has been described to be suitable for the multipoint covalent attachment of enzymes, permitting very high stabilizations [19]. The aldehydes from this support react with amino groups from the surface of the proteins to form reversible imino linkages. Therefore, proteins become immobilized on glyoxyl supports only through a simultaneous multipoint attachment [20] which normally occurs through the Lys richest region of the protein surface. After immobilization, a reduction with sodium borohydride yields a very inert surface (remaining aldehydes of the support are transformed into hydroxyl groups), and irreversible and very stable secondary amino bonds between the enzyme and the support are formed [21].

Considering that the first immobilizaition must be via a multipoint covalent attachment, enzyme immobilization on glyoxyl supports occurs very rapidly at alkaline pH values (pH values over 10.0) and using highly activated supports. However, proteins do not

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immobilize on poorly activated glyoxyl supports or at neutral pH, where only the terminal amino groups are unprotonated and, therefore, available to react with the glyoxyl groups [20]. As a result, in all examples found in the literature, the immobilization on glyoxyl agarose is performed at pH 10.0–10.1 [19].

Proteins having several terminal amino groups (e.g. proteins formed by several enzyme chains) might be an exception to this rule. The terminal amino groups have a p*K* in the range of pH 7.0–8.0, If they are placed in a position that permits their simulatenous interaction with a flat surface, this may permit enzyme immobilization on glyoxyl agarose even at a neutral pH. This has permitted to partially purify the tetrameric beta-galactosidase from *Escherichia coli*. The enzyme was selectively immobilized on glyoxyl agarose at pH 7.0, using poorly activated supports that rendered a low number of enzyme support imino bonds [22]. After this selective immobilization step, the purified enzyme was desorbed by adding other nucleophiles to give a soluble and pure enzyme [22].

Considering the good features of glyoxyl supports to stabilize proteins, immobilization of multimeric enzymes on glyoxyl agarose beads at neutral pH values could be coupled to the purification, immobilization and stabilization (via multi-subunit and multipoint covalent attachment) of the multimeric protein. For this purpose, after the first immobilization at neutral pH to immobilize mainly multimeric proteins with the terminal amino groups of several protein subunits located in the same plane, the immobilized proteins may be further incubated at alkaline pH for long periods of time to permit an intense multipoint covalent reaction between the enzyme and the support [23].

If this selectivity towards multimeric proteins of the first immobilization was in fact achieved, its maximum advantages should be obtained in the purification of a protein from a protein sample where the main multimeric enzyme was the target one. A multimeric thermophilic enzyme expressed in a mesophilic host would be an example of this situation, because after a thermal shock, most multimeric mesophilic proteins are destroyed [24,25].

Here, we present the results obtained after applying this strategy to the trimeric glutamate dehydrogenase (GDH) from *Thermus thermophilus* heterologously expressed in *E. coli* [26]. This new GDH exhibits a dual NAD/NADP activity while other hexameric NAD-specific GDH purified from cultures of *T. thermophilus* have already been described [27]. This GDH had been successfully stabilized by immobilization on glyoxyl agarose at pH 10 [26]. GDH may have interest in diverse areas, as design of biosensors [28–30] or as biocatalysts (for the production of keto-glutaric acid or as cofactor regenerator) [31–35].

2. Materials and methods

2.1. Materials

Nicotinamide adenine dinucleotide derivative (NAD^+) was purchased from Jülich Fine Chemicals (Jülich, Germany). Cyanogen bromide Sepharose 4-B 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 [19]. All other reagents were of analytical grade. Glutamate dehydrogenase from *T. thermophilus* was over expressed in *E. coli* and purified as published elsewhere [26]. Protein concentration was determined using Bradford's method [36].

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 L-glutamate oxidation ($\varepsilon = 6.22\ mM^{-1}$ at 340 nm). A sample of enzymatic preparation (25–400 μL) was added to a cell containing 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. When indicated, different temperatures and pH values were used. 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. Enzyme immobilization

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 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.

2.4. Immobilization on CNBr-activated sepharose 4-B

Immobilization was carried out by adding 2 g of the CNBr-activated support to 18 mL of 100 mM sodium phosphate at pH 7.0 containing 8 U of GDH. The suspension was kept under mild stirring for 15 min at 4 °C. The support was then filtered and washed with 100 mM sodium bicarbonate at pH 8.0 and incubated for 2 h in 1 M ethanolamine at pH 8.0 to block the remaining CNBr groups. Finally, the immobilized preparation was washed with distilled water.

2.5. Immobilization on glyoxyl agarose

2.5.1. Immobilization at pH 7.0

The immobilization was carried out by adding 2 g of support to 20 mL of 100 mM sodium phosphate pH 7.0 containing 8 U of soluble GDH. The suspension was gently stirred at 25 °C. In some cases, after enzyme immobilization, the derivative was vacuum filtered and incubated at 25 °C in 100 mM sodium bicarbonate at pH 10.05 for different times to increase the multipoint covalent attachment [23]. Finally, the immobilized preparations were reduced for 30 min at 25 °C with 20 mg sodium borohydride as previously described [19–21]. Then, the preparation was washed with 100 mM sodium phosphate at pH 7.0 and an excess of distilled water.

2.5.2. Immobilization at pH 10.0

The immobilization was carried out as previously described [26]: 2 g of support were added to 20 mL of 100 mM sodium bicarbonate pH 10.05 containing 8 U of soluble GDH. The suspension was gently stirred at 25 °C. After enzyme immobilization, the suspension was incubated at 25 °C for 1.5 h (optimal stabilization at pH 10 [26]). Finally, the immobilized preparations were reduced for 30 min at 25 °C with 20 mg sodium borohydride as previous described [19]. After this period of time the preparation was washed with an excess of distilled water and its activity assayed as described above.

2.6. Inactivation of different GDH preparations

Different GDH preparations (soluble and immobilized enzyme) were incubated at different temperatures and pH, or in the presence of acetone. Samples were withdrawn at different times, and residual activity was measured as previously described.

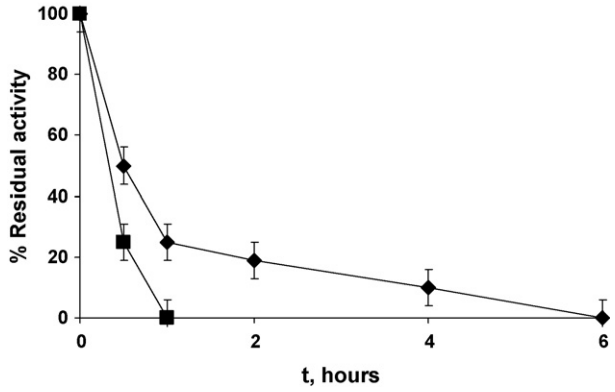

Fig. 1. Effect of the pH in the immobilization of GDH on glyoxyl agarose. (♦) Immobilization course at pH 7. (■) Immobilization course at pH 10. Experiments were performed at 25 °C.

2.7. SDS-PAGE analysis of GDH

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli [37]. The GDH derivatives were boiled in Laemmli's disruption buffer that contains mercaptoethanol and SDS, thus releasing any non-covalently bound protein from the support. Samples were analyzed in 12% polyacrylamide PAGE gels and stained using Coomassie blue.

3. Results

3.1. Immobilization of GDH in glyoxyl agarose beads under different conditions

Purified GDH [26] was incubated in the presence of glyoxyl agarose beads at pH 7.0 and 10.0. Fig. 1 shows that the enzyme becomes very rapidly immobilized at pH 10 (in 1 h there was no GDH in the supernatant). At pH 7, the enzyme also became quite rapidly immobilized on the support, after 6 h there was no GDH in the supernatant.

In order to permit an intense multipoint covalent attachment between the ε-amino groups of the Lys groups of the already immobilized enzyme and the glyoxyl groups of this support [19], the pH of the immobilization suspension of the enzyme immobilized at pH 7 was increased to 10.05 and incubated for a certain amount of time before reduction with sodium borohydride. Fig. 2 shows the effect of the reaction of the enzyme with the support on the GDH activity, comparing the evolution of the activity of the enzyme directly immobilized at pH 10 (by the area where there are most Lys groups) and the activity of the enzyme immobilized at pH 7 and then incubated at pH 10 for an equivalent period of time. The soluble enzyme remained fully active after 48 h. However, the activity of the enzyme immobilized at pH 10 decreased to around 15% of its original value after 24 h, while the enzyme immobilized at pH 7 retained around 50% of that activity under similar conditions and over 40% after 48 h. This decrease in activity should be produced by the reaction between the enzyme and the support, because the soluble enzyme remained fully active at pH 10 [26]. The different effect of the incubation on the enzyme activity could be due to different reasons. For example, the orientation of the enzyme could be different in both cases. At pH 10, the richest area in amino groups should drive the protein immobilization [20], whereas at pH 7, the area of the enzyme having more terminal amino groups could play the major role [22]. Another likely explanation could be that the immobilization of the enzyme at pH 7 keeps the assembly of the enzyme subunits unaltered; therefore a further multipoint covalent attachment could produce lower enzyme distortion.

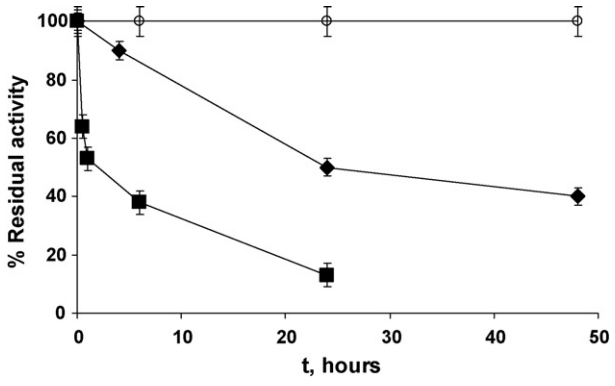

Fig. 2. Effect of the incubation at pH 10 on the activity of GDH immobilized on glyoxyl agarose at different pH values. (♦) GDH-glyoxyl agarose immobilized at pH 7. (■) GDH-glyoxyl agarose immobilized at pH10.0). (○) Soluble GDH. Incubation was performed at 25 °C as described in Section 2.

In any case, the immobilization of GDH on glyoxyl agarose beads at pH 7 permits to retain higher activities values than the direct and conventional immobilization at pH 10 (Fig. 2). This result suggested that the enzyme was more distorted when the enzyme–support reaction involved the area of the protein implied in the immobilization at pH 10.

The K_m for L-glutamate and NAD^+ increased after the immobilization protocol developed in this paper, suggesting some slight distortion of the enzyme structure (from 3.0 ± 0.5 to 4.0 ± 0.5 mM and from 0.020 ± 0.005 to 0.035 ± 0.008 mM, respectively).

3.2. Stability of the different GDH preparations

3.2.1. Structural stabilization of GDH

The number of enzyme subunits involved in the immobilization on glyoxyl agarose was analyzed by SDS-PAGE of the supernatant obtained after boiling the immobilized enzyme in the presence of SDS and mercaptoethanol to release any non-covalently attached subunit to the support. For this purpose, the high stability of the secondary bonds obtained after reduction with sodium borohydride was utilized: only protein subunits not attached to the support could appear in the SDS gel [37,38]. None of the glyoxyl preparations released enzyme subunits to the medium after this treatment, suggesting that all the enzyme subunits were immobilized to the support in all cases. Therefore, in both cases, enzyme

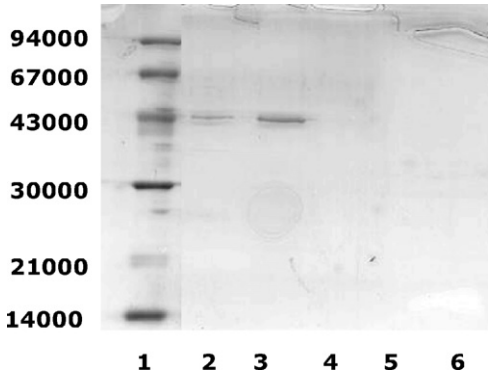

Fig. 3. SDS-PAGE analysis of the proteins released from different GDH preparations. Lane 1: molecular weight markers; Lane 2: soluble enzyme; Lane 3: CNBr agarose GDH derivative; Lane 4: glyoxyl agarose GDH immobilized pH 7; Lane 5: glyoxyl agarose derivative immobilized at pH 7 and then incubated at pH 10 for 24 h; Lane 6: glyoxyl agarose derivative directly immobilized at pH 10 and incubated at pH for 1.5 h.

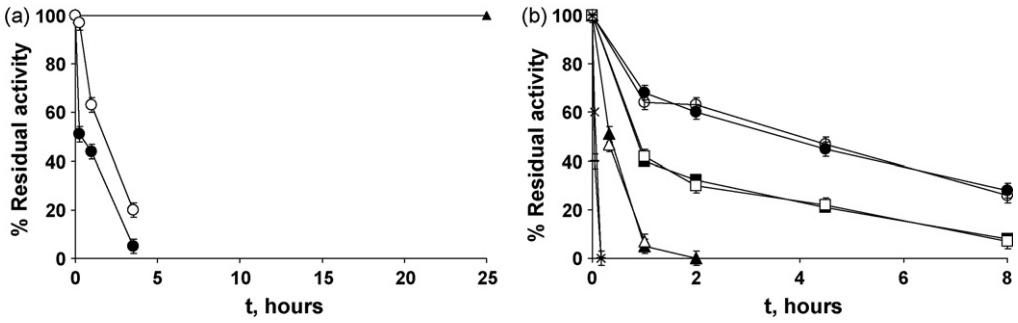

Fig. 4. Inactivation courses of different GDH preparations at pH 4. (a) At 25 °C (▲) GDH immobilized on glyoxyl agarose at pH 7 and immediately reduced (0.06 U/mL); (●) GDH-CNBr 0.06 U/mL; (O) GDH-CNBr-agarose (0.3 U/mL). (b) At 58 °C GDH immobilized on glyoxyl agarose at pH 7 and then incubated at pH 10 for 24 h using a GDH concentration of 0.06 IU/mL) (●) or 0.3 IU/mL (○); GDH immobilized on glyoxyl agarose at pH 10 and incubated for 1.5 h before reduction using a GDH concentration of 0.06 IU/mL (■) or 0.3 IU/mL (□); GDH immobilized on glyoxyl agarose at pH 7 and immediately reduced using a GDH concentration of 0.06 IU/mL (▲) or 0.3 IU/mL (△); CNBr-GDH using a GDH concentration of 0.06 IU/mL (–) or 0.3 IU/mL (✳). Experiments were carried out as described in Section 2.

dissociation was not detected. On the contrary, CNBr-GDH released to the medium some enzyme subunits after this treatment, suggesting that some subunits could desorb from the support [26] (Fig. 3).

Next, the different enzyme preparations were incubated at pH 4 and 25 °C, conditions where the main reason for GDH inactivation is enzyme dissociation [26]. The only preparation that was inactivated (very rapidly) was the CNBr-GDH preparation, and the course of inactivation depended strongly on the GDH concentration; while all other preparations remained fully active after 24 h (Fig. 4a). The increase in temperature to 58 °C (Fig. 4b) shows the effect of the multipoint covalent attachment of each of the monomers of the enzyme with the support on the enzyme stability. The stability of the enzyme preparation immobilized at pH 7 and incubated at pH 10 was much higher than the stability of the enzyme just immobilized at pH 7. The stability of this preparation was significantly higher than that of the enzyme directly immobilized at pH 10 (the half life increases by a factor of 2.5-fold) [26]. Moreover, the inactivation rate was not dependent on the enzyme concentration, as was expected from the structural stabilization of the quaternary structure of the enzyme.

The stabilities of the three preparations were also evaluated under other conditions where the dissociation of the enzyme subunits has been described to be not so relevant [26].

At pH 7 and 70 °C (Fig. 5), the enzyme just immobilized at pH 7 was significantly more stable than the CNBr-GDH preparation. However, the incubation at pH 10 before reduction permitted to significantly improve the stability of the enzyme immobilized at pH 7. This preparation retained 80% of its activity after 80 h while the enzyme just immobilized at pH 7 lost more than 80% of its activity under similar conditions. This suggests that both the prevention of enzyme subunits dissociation and the rigidification of each monomer of the enzyme are important to improve the stability of the enzyme. The enzyme directly immobilized at pH 10 presented slightly lower stability than the preparation obtained by immobilization at pH 7 followed by incubation at pH 10.

The effect of acetone on the enzyme stability was also studied. The use of immobilized enzymes prevented any risk of enzyme precipitation. In the presence of acetone (Fig. 6), the most stable preparation was again that obtained by immobilization at pH 7 and further incubated at pH 10, which retained over 90% of activity after 75 h of incubation. In contrast, the preparation obtained by immobilization at pH 10 retained 80% of the activity and the enzyme just immobilized at pH 7, retained around the 15% of the activity.

3.3. *Purification of the enzyme by immobilization at pH 7 on glyoxyl agarose beads*

To check the feasibility of this protocol to purify the GDH, the crude extract containing the enzyme was submitted to a thermal shock to destroy all multimeric proteins from the mesophilic host [24,25]. After the thermal shock, the protein sample was centrifuged to discard the precipitate and the supernatant was incubated with glyoxyl agarose beads at pH 7, and the immo-

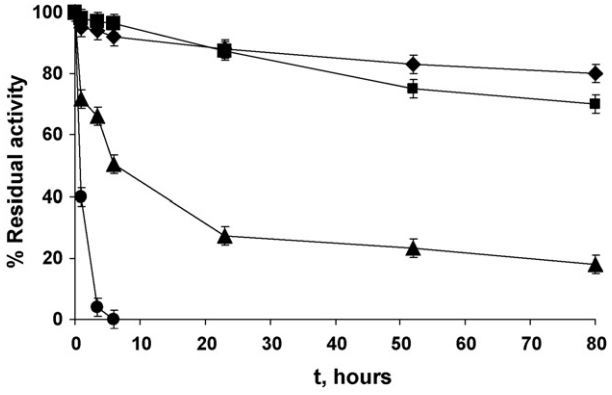

Fig. 5. Inactivation courses of different preparations of glutamate dehydrogenase at pH 7. (♦) GDH immobilized on glyoxyl agarose at pH 7 and then incubated at pH 10 for 24 h. (■) GDH immobilized on glyoxyl agarose at pH 10 and incubated for 1.5 h before reduction (▲) GDH immobilized on glyoxyl agarose at pH 7 and immediately reduced (●) CNBr-GDH. Experiments were carried out at 70 °C as described in Section 2, using 0.06 IU/mL.

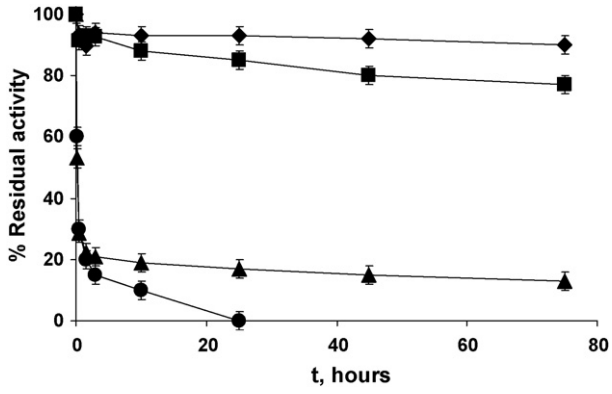

Fig. 6. Inactivation courses of different GDH preparation in presence of acetone. (♦) GDH immobilized on glyoxyl agarose at pH 7 and then incubated at pH 10 for 24 h (■) GDH immobilized on glyoxyl agarose at pH 10 and incubated for 1.5 h before reduction (▲) GDH immobilized on glyoxyl agarose at pH 7 and immediately reduced (●) CNBr-GDH. Experiments were carried out in 50% 100 mM Tris–HCl pH 7/50% acetone (V/V).

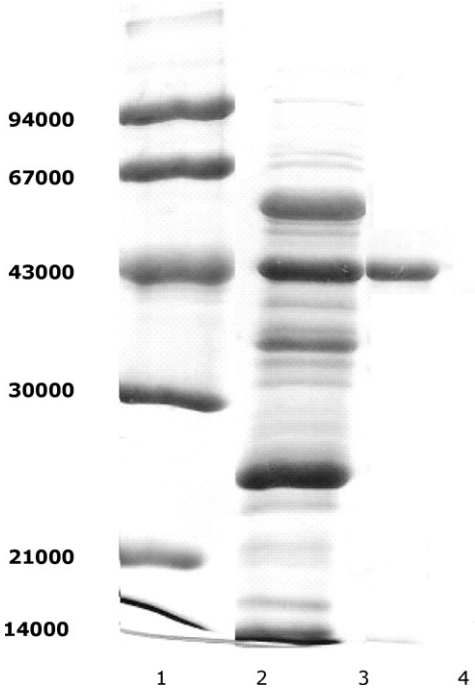

Fig. 7. SDS-PAGE analysis of the proteins released from different GDH preparations. Lane 1: molecular weight markers; Lane 2: supernatant of the crude extract after thermal shock; Lane 3: glyoxyl agarose pH 7 before reduction; Lane 4: glyoxyl agarose pH 7 reduced.

bilized fraction (without the reduction step) was washed and re-suspended in 1 M ethanolamine at pH 9 in the presence of SDS and mercaptoethanol and boiled for 10 min to desorb all the proteins immobilized on the support (the imino bonds can be broken under these conditions) [20,21]. The supernatants analyzed by SDS-PAGE (Fig. 7) showed that the immobilization of GDH at pH 7, while it did not permit the complete purification of the enzyme, it yielded very high purification factors, as more than 90% of the proteins detected by Coomassie staining corresponded to GDH. The small percentage of other proteins detected could be proteolyzed enzymes. Proteolysis may generate some additional terminal amino groups, and these enzymes could become immobilized on glyoxyl agarose at neutral pH value [22]. Nevertheless, the final purification coupled to the immobilization seems very adequate for most industrial purposes. When the immobilization was performed at pH 10, more than 95% of the proteins contained in the crude extract were immobilized on the glyoxyl support (as previously reported) [22].

3.4. Conclusions

The results presented herein showed that GDH could be immobilized at pH 7 on glyoxyl agarose. This is an exception and only because of the multimeric nature and the coincidence that at least two of the amino groups are in a plane (results suggest that all three are in the same plane). This permit that after heating the protein sample (the enzyme is produced in *E. coli*) to destroy other multimeric enzymes, GDH may become immobilized on the support at pH 7, and using a crude extract become purified during the immobilization.

The enzyme immobilized at pH 7 presented all the enzyme subunits attached to the support, in this way the enzyme was not readily inactivated by incubation at acidic pH as occurred with the soluble enzyme because the enzyme dissociation was no longer possible. However, if the immobilized enzyme at pH 10 was incubated at pH 10, the rigidification of each monomer structure permitted to greatly improve the overall enzyme stability, very likely by multipoint covalent attachment of each monomer [19,23].

The results obtained with this enzyme in terms of recovered activity and stabilization by multipoint covalent attachment were better when the first immobilization was performed at pH 7 instead of the conventional immobilization on glyoxyl agarose at pH 10 [26].

The immobilization of the GDH on glyoxyl agarose beads at pH 7 permitted to purify the enzyme by selective immobilization, while glyoxyl agarose can immobilize most proteins at pH 10. This means that now it is not necessary to use a purified enzyme to have a highly loaded biocatalyst, since immobilization and purification are coupled The use of glyoxyl agarose beads as a chromatographic matrix to purify enzymes seemed to be complex, because it was difficult to release the enzyme from highly activated glyoxyl agarose beads [22]. However, now it has been shown that, by coupling the immobilization and stabilization of the enzyme to its purification, the immobilization of multimeric enzymes on glyoxyl agarose at neutral pH values seems to be a very promising approach to prepare industrial biocatalysts not just of this very interesting enzyme, but also of many other multimeric enzymes of industrial interest.

This is the first report that shows how a multimeric enzyme may be purified, immobilized and stabilized using glyoxyl agarose by immobilization and incubation under proper conditions.

Moreover, the results shown in this paper shows that a new orientation of a multimeric enzyme on the glyoxyl-support may be obtained. This is important bearing in mind that the orientation of the enzymes on the support may affect, the biocatalyst features (different activity recovery, different activity against macromolecular substrates, different stabilization or different selectivity) [1].

Acknowledgments

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