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Immobilization–stabilization of a new recombinant glutamate dehydrogenase from *Thermus thermophilus*

Juan M. Bolivar · Felipe Cava · Cesar Mateo · Javier Rocha-Martín · Jose M. Guisán · José Berenguer · Roberto Fernandez-Lafuente

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Abstract The genome of *Thermus thermophilus* contains two genes encoding putative glutamate dehydrogenases. One of these genes (TTC1211) was cloned and overexpressed in *Escherichia coli*. The purified enzyme was a trimer that catalyzed the oxidation of glutamate to α-ketoglutarate and ammonia with either NAD^+ or $NADP^+$ as cofactors. The enzyme was also able to catalyze the inverse reductive reaction. The thermostability of the enzyme at neutral pH was very high even at 70°C, but at acidic pH values, the dissociation of enzyme subunits produced the rapid enzyme inactivation even at 25°C. The immobilization of the enzyme on glyoxyl agarose permitted to greatly increase the enzyme stability under all conditions studied. It was found that the multimeric structure of the enzyme was stabilized by the immobilization (enzyme subunits could be not desorbed from the support by boiling it in the presence of sodium dodecyl sulfate). This makes the enzyme very stable at pH 4 (e.g., the enzyme activity did not decrease after 12 h at 45°C) and even improved the enzyme stability at neutral pH values. This immobilized enzyme can be of great interest as a biosensor or as a biocatalyst to regenerate both reduced and oxidized cofactors.

Keywords Redox enzymes · Thermophilic enzymes hyperstabilization · Multipoint immobilization · Multimeric enzyme stabilization · Cofactor regeneration · Glutamate biosensor

J. M. Bolivar · C. Mateo · J. Rocha-Martín · J. M. Guisán (✉) · R. Fernandez-Lafuente
Departamento de Biocatálisis. Instituto de Catálisis y Petroleoquímica, CSIC, Campus UAM—Cantoblanco, 28049 Madrid, Spain
e-mail: jmguisan@icp.csic.es

R. Fernandez-Lafuente
e-mail: rfl@icp.csic.es

F. Cava · J. Berenguer
Centro de Biología Molecular Severo Ochoa (CSIC-UAM), Campus UAM—Cantoblanco, 28049 Madrid, Spain

J. Berenguer
e-mail: jberenguer@cbm.uam.es

Introduction

NADP(H)-specific glutamate dehydrogenase (GDH) enzymes usually catalyze the assimilation of ammonia by reductive amination of α-ketoglutarate to form L-glutamate (anabolic), while NAD(H)-dependent GDH enzymes catalyze the reverse reaction (catabolic; Joe et al. 1994; Reitzer and Magasanik 1987; Smith et al. 1975). Microbial GDHs could be classified as three types according to the coenzyme used: NADP dependent (EC 1.4.1.4; Alen et al. 1997; Ha et al. 2003; Yip et al. 1998), NAD dependent (EC 1.4.1.2; Kobayashi et al. 1995; Molitor et al. 1998; Knapp et al. 1997), and dual-coenzyme specific (EC 1.4.1.3), the latter GDH being most frequently found among higher eukaryotes (Lebbink et al. 1998; Rahman et al. 1998).

Dehydrogenases have a great interest in organic chemistry as an asymmetric redox catalyst (Hummel 1997, 1999), but these enzymes require the consumption of a expensive cofactor (NAD(H) or NADP(H)) in stoichiometric amounts. To overcome this problem, additional redox enzymes can be coupled to these reactions to recycle the cofactors (Leonida 2001; Van der Donk and Zhao 2003; Zhao and Van der Donk 2003). GDHs may be a suitable solution for this goal (Chenault et al. 1988; Van der Donk

and Zhao 2003; Zhao and Van der Donk 2003). Another technological use of GDH is as a biosensor (Basu et al. 2006; Doong and Shih 2006; Bertocchi et al. 1996; Rodriguez et al. 2004a, b).

The use of GDH from thermophilic microorganisms may have some advantages for both applications because of their higher stability (Daniel and Cowan 2000; Jaenicke et al. 1996; Cowan 1997; Owusu and Cowan 1989). Recently, the genes coding for the GDHs from several thermophilic and hyperthermophilic archaea and bacteria have been sequenced, and the encoded proteins have been characterized (Maras et al. 1992, 1994; Britton et al. 1995; Higuchi et al. 1997; Díaz et al. 2006; Hudson et al. 1993; Kort et al. 1997; Kujo and Ohshima 1998; Lebbink et al. 1999; Robb et al. 2001).

However, the previous immobilization of the enzymes, even if the enzymes are going to be trapped in ultrafiltration reactors (Hummel 1997, 1999; Wandrey 2004; Liu and Wang 2007) or in biphasic systems (Eckstein et al. 2004), may have some advantages (Bolivar et al. 2006a, b).

Similarly, enzymes are usually immobilized when they are going to be used in biosensors, usually by trapping the enzyme on a polymer on the tip of the sensor (Bertocchi et al. 1996; Rodriguez et al. 2004a,b; Basu et al. 2006; Doong and Shih 2006). In this case, the preimmobilization of the enzyme on porous supports also presents some advantages (Betancor et al. 2005). Obviously, the advantages of using preimmobilized enzymes will be much more evident if the immobilization permit its stabilization, e.g., via multipoint or multisubunit covalent attachment (Gupta 1991; Klibanov 1979, 1983; Mateo et al. 2007). There are many examples in the literature showing that the stability of thermophilic enzymes may be further improved by using proper immobilization techniques (Nakkharat and Haltrich 2006; Nawani et al. 2006; Pessela et al. 2003; Tardioli et al. 2006).

Glyoxyl agarose supports are one of the most effective supports to stabilize proteins (Mateo et al. 2005, 2006). This support has been utilized to highly stabilize many different enzymes with high-activity recovery values (Mateo et al. 2006).

In the literature, a hexameric NAD-specific GDH purified from cultures of *Thermus thermophilus* HB8 has been described (Ruiz et al. 1998, 2003), although no correspondence with specific genes among the published sequence of this strain has been described (http://wishart.biology.ualberta.ca/BacMap). In this paper, we show that one of these genes, with codes TTC1211, produces an active NAD/NADP-dependent bifunctional GDH when expressed in *Escherichia coli*. We also describe immobilized and highly stabilized biocatalysts for its use either as cofactor regenerator in biotransformation or in biosensors involving redox reactions.

Experimental section

Materials Nicotinamide adenine dinucleotide derivatives (NAD^+, NADH, $NADP^+$), were purchased from Jülich Fine Chemicals. Cyanogen bromide 4 B Sepharose was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Crosslinked agarose beads (6%) were from Amersham Biosciences. Glutamic acid and α-ketoglutaric acid were supplied by Sigma-Aldrich Chemicals. Glyoxyl agarose beads were prepared as previously described (Guisán 1988). All other used reagents were of analytical grade.

Bacterial strains, plasmids, and growth conditions T. thermophilus HB27 was a generous gift from Dr. Koyama. The *E. coli* strains DH5αF′ (F′, supE44, Δ[lacZYA-argF] U169 [Φ80 lacZΔM15] hsdR17 recA1 endA1 gyrA96 thi I relA1; Bethesda Research Laboratories, Gaithersburg, MD, USA) and BL21DE3 (*hsd*S, *gal* [λ*c*Its857, *ind*1, *Sam7*, *nin5*, *lacUV5*-T7 gene 1]) were used for cloning purposes and protein expression, respectively. Plasmid pET28b (Novagen) was used for the expression of the *gdh* gene in the BL21DE3 strain. *T. thermophilus* was grown at 70°C under strong aeration, in TB medium (Lasa et al. 1992). *E. coli* was routinely grown at 37°C in Luria–Bertani liquid medium or on plates. Kanamycin (30 mg/l) was added for plasmid selection when needed.

Cloning, expression, and purification of the GDH Total deoxyribonucleic acid (DNA) from *T. thermophilus* was purified following standard methods (Sambrook et al. 1989). A putative GDH gene (*GDH*), identified in the genome of *T. thermophilus* HB27 with code TTC1211 (http://wishart.biology.ualberta.ca/BacMap), was amplified from this DNA by polymerase chain reaction (PCR) with primers gdh2sense (5′-cg*catatg*aagagcgaa-3′), which included an *Nde*I restriction site in the forward primer (italicized) for further cloning purposes, and gdh2antisense (5′-ttaagggtataggcccc-3′) as reverse primer. The gene was firstly cloned into the pCR2.1TOPO vector (Invitrogen) and then into plasmid pET28b to get the pET28gdh2 construct, in which the putative GDH should be expressed with an N-terminal (6×)-histidine tag. The *gdh* insert of this construct was sequenced to confirm the absence of mutations.

For the overexpression of the *gdh* gene, this plasmid was used to transform the BL21DE3 strain, and exponential cultures of the transformants were treated with isopropyl-β-D-thiogalactopyranoside (1 mM) when they reached an optical density at 550 nm of 0.5 and further incubated for 4 h under the same conditions. After cooling to 4°C, the cells were collected by centrifugation (10,000×*g*, 10 min), resuspended in a 1/100 volume of 50 mM Tris–HCl, 50 mM NaCl pH 8 buffer, and disrupted by mechanical

abrasion with alumina (0.5 g/mL). The soluble fraction obtained after centrifugation (20,000×*g*, 15 min, 4°C) was used as source of the GDH in further purification steps.

The purification of the GDH was carried out in two steps; first, the most *E. coli*-contaminant proteins were denatured by incubation at 70°C for 30 min and eliminated by centrifugation. In a second step, we used Ni–agarose affinity chromatography and specific elution with imidazole to get a greater than 99% pure protein.

Protein characterization The amount of protein of the soluble enzymatic preparation was determined by the method developed by Bradford (1976). The analysis of the molecular size of the enzyme was carried out by gel filtration and ultracentrifugation. Gel filtration was carried out on Sephacryl S-200 (Pharmacia) columns (85×2 cm) with phosphate-buffered saline (PBS) buffer (50 mM phosphate, 50 mM NaCl, pH 7.5), at a flow rate of 0.5 mL/min. The enzymes pyruvate kinase from rabbit muscle (237 kDa, SIGMA), Glucose oxidase from *Aspergillus niger* (160 kDa, Boehringer), and Neuraminidase (43 kDa, SIGMA) were used as chromatographic internal controls. Ultracentrifugation was carried out in an Analytical Ultracentrifugation and Macromolecular Interactions facility of the Centro de Investigaciones Biológicas (CSIC, Madrid) with a 3 mg/mL GDH solution in PBS buffer.

Enzyme assays, kinetic parameters, substrates specificity The activities of the different GDH preparations were analyzed by the increase in absorbance at 340 nm corresponding to the formation of NAD(P)H 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 $NAD(P)^+$ in 100 mM sodium phosphate at pH 8.0 and 66°C. When indicated, different temperatures and pH values were used. The activity toward α-ketoglutarate was measured as absorbance decrease at 340 nm due to NAD(H) oxidation. A sample of the enzymatic preparation (25–400 μL) was added to 2 mL of 250 mM α-ketoglutaric acid and 100 μL of 10 mM NADH in 100 mM ammonium phosphate and 66°C. 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 GDH preparation used for immobilization assays had a specific activity of 2.45 U/mg of protein and 1.5 U/mL.

Kinetic parameters were calculated measuring initial velocity (Cleland 1967, 1977). The reaction was initiated by adding GDH to the reaction mixture. NADH–GDH activity was assayed for the reductive amination reaction at different concentrations of α-ketoglutarate, NADH, or ammonium chloride and at saturating concentrations of the remaining substrates (50 mM α-ketoglutarate, 250 μM NADH, and 50 mM ammonium chloride). For the deamination oxidative reaction, different concentrations of glutamate or $NAD(P)^+$ and saturating concentration of the remaining substrate (50 mM glutamate and 250 μM NAD $(P)^+$) were used. Measurements were performed at 66°C in 100 mM sodium phosphate at pH 8. Triplicate assays were performed at each concentration. Results were fitted using linear regression analysis of the data from each fixed concentration.

Immobilization of the enzyme 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 the protein concentration were assayed. All the experiments were performed using less than 4 U per milliliter of support to avoid diffusion problems that could alter the apparent enzyme stability.

Immobilization on CNBr-activated sepharose 4 B 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 or at 25°C. After, the support was filtered and washed with 100 mM sodium bicarbonate buffer at pH 8 and incubated for 2 h in 1 M ethanolamine at pH 8 to block the remaining CNBr groups. Finally, the immobilized preparation was washed with distilled water.

Immobilization on glyoxyl agarose The immobilization was carried out by adding 2 g of support to 20 mL of 100 mM sodium bicarbonate pH 10.05 containing 8 U of soluble GDH. The suspension was gently stirred at 4°C or 25°C. After the enzyme immobilization, the suspension was incubated at 25°C for different times to increase the multipoint covalent attachment. Finally, the immobilized preparations were reduced for 30 min at 25°C with 20 mg sodium borohydride as previously described (Blanco and Guisán 1989). After this time, the preparation was washed with an excess of distilled water and assayed.

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

SDS-PAGE analysis of GDH Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) was

performed according to Laemmli (1970). The GDH derivatives were boiled in Laemmli's disruption buffer that contains mercaptoethanol and SDS, thus releasing from the support any noncovalently bound protein. Samples were analyzed in 12% PAGE gels and detected by Coomassie blue staining.

Native PAGE analysis of GDH For native PAGE, an 8% polyacrylamide gel was developed in the absence of SDS with the same buffer system. The detection of the activity in situ was carried out at 37°C in the dark in 50 mL of 50 mM Tris–HCl buffer and 2 mM ethylenediamine tetraacetic acid, pH 8, containing 3 mM NAD^+ (or $NADP^+$), 0.1 M L-glutamate, 15 mg nitroblue tetrazolium, and 1 mg phenazine methosulfate. After incubation, the gel was rinsed in water and fixed in ethanol/acetic acid/glycerol/water (5:2:1:4).

Influence of temperature and pH on the activity of immobilized GDH The activity of soluble and glyoxyl agarose-immobilized GDH preparations was assayed at different temperatures with glutamic acid (250 mM) in 100 mM sodium acetate, pH 5. The activity was measured during 0 to 150 s of the reaction.

The influence of the pH value on the optimal GDH preparations was analyzed by measuring their activities in the oxidation of glutamate with NAD^+ and $NADP^+$ and in the reduction of α-ketoglutarate with NADH, in 100 mM of sodium or ammonium phosphate at the appropriate pH. The expressed activity was measured during the first 50 s of the reaction.

Results

Cloning and expression of GDH from *Thermus thermophilus* in *E. coli*

A gene encoding a putative GDH of *T. thermophilus* HB27 (code TTC1211 in http://wishart.biology.ualberta.ca/BacMap) was amplified by PCR and cloned in an expression vector to get plasmid pET28gdh. Its expression in *E. coli* produced a protein band of the size expected for the molecular mass of the putative protein deduced from the sequence of the TC1211 gene (around 46 kDa; Fig. 1a). Taking advantage of its probable thermostability and the presence of a N-terminal His-tag, the protein was subsequently purified from the soluble fraction by heat treatment and affinity chromatography on Ni^{2+}–agarose columns, from which it was detached by the addition of increasing concentrations of imidazole (Fig. 1b). A first assay of activity at 70°C revealed that the protein reduced NAD^+ in the reaction:

$$\text{L} - \text{glutamate} + \text{NAD}^+ + \text{H}_2\text{O} \rightarrow \alpha - \text{ketoglutarate} + \text{NADH} + \text{H}^+ + \text{NH}_3$$

(Fig. 1c).

SDS-PAGE showed a band of around 42 kDa. The putative oligomeric nature of this enzyme was studied by gel filtration and by analytical ultracentrifugation. The first method revealed that most GDH activity eluted at the retention volume supporting a trimeric nature for the GDH, detecting some minor larger aggregates (results not shown).

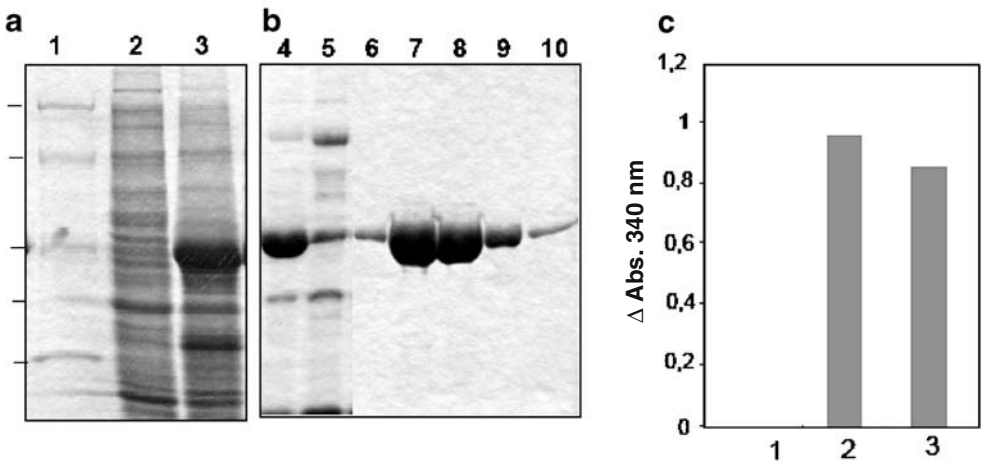

Fig. 1 Expression, purification and enzymatic activity of GDH. **a** Expression of GDH in *E. coli*. *1*, Protein size markers (115.9, 96.7, 51.5, 37.7, 29.2); *2*, *E. coli* transformed with pET28b; *3*, *E. coli* transformed with pET28gdh. **b** Purification of GDH. *4*, Fraction obtained after heating, *5* flow through; *6–10*, fractions eluted with imidazole: 20, 50, 100, 300, and 500 mM. **c** GDH activity detected by the increase in absorbance at 340 nm due to NAD^+ reduction. *1*, Soluble fraction of *E. coli* transformed with pET28 after heating at 70°C; *2*, *3*, purified fractions corresponding to lanes *7* and *8* of **b**, respectively

On the other hand, analytical ultracentrifugation revealed a sedimentation peak corresponding to 5.2S, which is also compatible with a trimeric structure for this enzyme. However, additional sedimentation peaks were detected at 8.4S, which approximately corresponds to twice the size of the first peak, and even larger complexes (11.6S) were also detected. These data suggest that the minimal active unit for GDH is most likely a trimer and that the protein can form higher-order aggregates at the high-protein concentrations used in analytical centrifugation (3 mg/mL).

Kinetic parameters, substrates specificity

The purified enzyme was assayed for its capability to use L-glutamate, α-KG, and D-glutamate as substrates, finding no activity for the latter substrate (data not shown). On the other hand, NAD^+ and $NADP^+$ were also tested as putative cofactors in a zymogram gel electrophoresis, using glutamic acid as the substrate. The enzyme was able to use either NAD^+ or $NADP^+$ in the above reaction but with lower activity with the latter cofactor (results not shown). Thus, in contrast to the first GDH described from *T. thermophilus* (Ruiz et al. 1998, 2003), this enzyme has a dual-cofactor activity, which is rare among prokaryotic GDHs.

Calculated kinetic parameters were Km (L-glutamate)=3.0±0.5 mM, Km (NAD+)=0.020±0.005 mM, Km ($NADP^+$)=0.035±0.005 mM, Km (α-ketoglutarate)=3.5±0.5 mM, Km (NADH)=0.040±0.005 mM, and Km (ammonium)=10±2 mM.

Immobilization of the recombinant enzyme

Different immobilized enzyme preparations were prepared. Initially, soluble GDH was immobilized under mild conditions onto CNBr agarose and glyoxyl agarose. Although we could immobilize up to 50 mg of enzyme per gram of agarose 6BCL, all studies were performed using lowly loaded preparations as indicated in methods to study the intrinsic properties of each individual enzyme molecule, without having diffusion limitations.

The immobilization onto CNBr agarose at 4°C for 15 min permits the recovering of more than 95% offered enzymatic activity. This method is very mild and mainly immobilizes the enzyme by the most reactive amino groups (e.g., the terminal one; Mateo et al. 2005). The glyoxyl agarose derivative, prepared at 4°C for 15 min, only immobilized around 50% of the enzyme, but the enzyme retains almost full activity after the immobilization (Table 1).

All immobilized preparations, as well as the soluble enzyme, remained fully active after 24 h of incubation at 60°C and pH 7. An increase in the incubation temperature up to 66°C produces a significant time-dependent decrease in the activity on this time period. At temperatures higher than 70°C, the stability decreases dramatically. Figure 2 shows the inactivation courses of the different enzyme derivatives at 72°C. The glyoxyl agarose–GDH immobilization was more stable than the CNBr–agarose preparations or than the soluble enzyme.

Table 1 Immobilization yield and expressed activity of different GDH-immobilized preparations

Derivatives	Immobilization yield (%)	Expressed activity (%)
BrCN–agarose, 4°C, 15 min	100	>95
BrCN–agarose, 25°C, 6 h	100	90
GA 6BCL, 75 μmol/g, 15 min, 4°C	50	90
GA 6BCL, 10 μmol/g, 25°C	8	100
GA 6BCL, 30 μmol/g, 25°C	100	90
GA 6BCL, 75 μmol/g, 30 min, 25°C	100	51
GA 6BCL, 75 μmol/g, 1.5 h, 25°C	100	46
GA 6BCL, 75 μmol/g, 6 h, 25°C	100	31

Optimization of the enzyme stabilization by immobilization

The enzyme immobilized on CNBr–agarose was left to interact with the support for longer times at 25°C before chemical blockage of the support. This incubation did not promote any improvement of the stability at 72°C (data not shown). On the other hand, different glyoxyl agarose derivatives were prepared by controlling the immobilization conditions: temperature, reaction time, and activation degree of the supports. These variables have been reported to control the intensity of the enzyme–support reaction and therefore their stability and activity (Pedroche et al. 2007).

Table 1 shows the immobilization yield and activity recovery using different degrees of glyoxyl activation. Using a support with 10 μmol glyoxyl groups per gram,

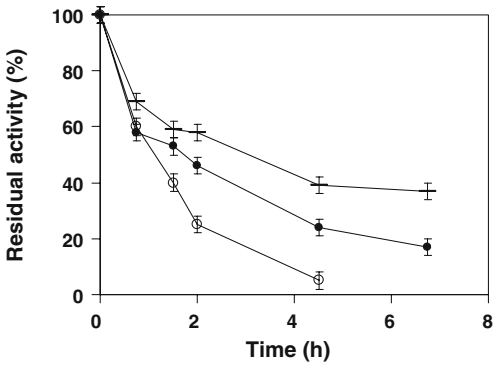

Fig. 2 Inactivation courses of different preparations of glutamate dehydrogenase. Experiments were carried out at 72°C and pH 7, in sodium phosphate 100 mM. Symbols: *empty circles*, soluble GDH, 0.3 U/mL; *filled circles*, CNBr derivative 4°C 15 min; *bar*, glyoxyl agarose derivative 4°C, 15 min

only a small percentage (8%) of the enzyme became immobilized, but the activity of the immobilized enzyme was fully retained. If the activation of the support was 30 μmol/g, all the enzyme became immobilized after 6 h, and the activity recovery was around 90%, while supports bearing 75 μmol/g were able to immobilize the enzyme after only 30 min, with an activity decrease to 50%. Figure 3 shows the inactivation courses at 72°C of these preparations. The most stable preparation was that obtained using the most activated support at 25°C. Lower activation degrees in the support improved the recovered activity, but the stability decreased. In fact, the lowest activated support produced enzyme preparations with similar stability to that of the soluble enzyme.

After 30 min at 25°C, using the support activated with 75 μmol/g of glyoxyl agarose, all the enzyme activity was immobilized. However, to obtain a more intense reaction between the support and the enzyme, we left the enzyme reacting with the support before reducing the remaining aldehyde groups (Pedroche et al. 2007). Table 1 shows that longer reaction times determine a slight decrease in the expressed activity; however, it promoted a significant increase in the stability (Fig. 4) up to 1.5 h of incubation; longer incubation times did not increase enzyme stability but still produce a decrease in enzyme activity.

The stability of the enzyme and the immobilized biocatalysts in the presence of acetone was also studied. The soluble enzyme remains fully active in the presence of 25% *v*/*v* acetone at pH 7 and 25°C for 1 week. However, higher concentrations of acetone produced a rapid inactivation of the enzyme. In 50% *v*/*v* of acetone, the half-life of CNBr preparation was less than 4 min. Nevertheless, the glyoxyl agarose preparation presented a significant stabilization, retaining more than 70% of its activity after 3.5 h under identical conditions.

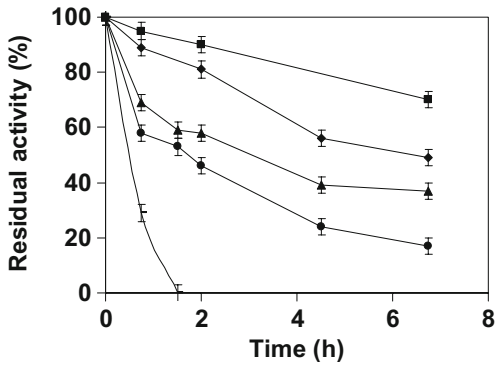

Fig. 3 Influence of reaction temperature, reaction time, and activation degree of support of glyoxyl agarose derivatives in thermal inactivation courses. Experiments were carried out at 72°C in sodium phosphate at pH 7. Symbols: *bar*, glyoxyl agarose, 10 μmol/g; *circles*, CNBr agarose; *triangles*, glyoxyl agarose, 75 μmol/g, 4°C, 15 min; *diamonds*, glyoxyl agarose, 30 μmol/g, 25°C; *squares*, glyoxyl agarose, 75 μmol/g, 25°C, 30 min

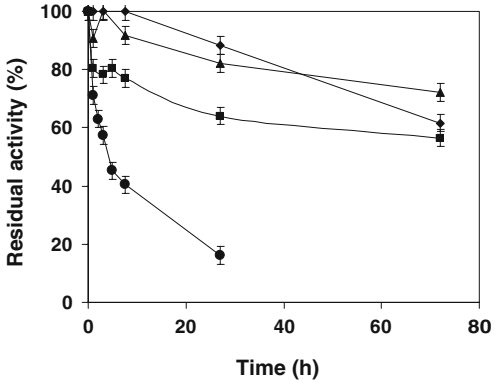

Fig. 4 Inactivation courses of different glyoxyl agarose derivatives. Experiments were carried out at 66°C and pH 7, in sodium phosphate 100 mM. Symbols: *circles*, CNBr derivative; *squares*, glyoxyl agarose, 75 μmol/g, 25°C, 30 min; *triangles*, glyoxyl agarose, 75 μmol/g, 25°C, 1.5 h; *diamonds*, glyoxyl agarose, 75 μmol/g, 25°C, 6 h

Thus, the optimal glyoxyl agarose derivative was prepared onto glyoxyl agarose activated with 75 μmol/g and with an enzyme–support reaction time of 1.5 h at 25°C. This preparation retains 80% of the activity after 60 h under conditions where the CNBr preparation was fully inactivated in 40 h and also was much more stable in the presence of acetone. As conditions that favor the multipoint covalent attachment improve the stabilization observed (Pedroche et al. 2007), this increase in the enzyme support bonds should be the most likely explanation for the observed results.

Stability at acid pH

The stability of this thermophilic enzyme at pH 4 was quite low and decreased when decreasing the enzyme concentration (Fig. 5a) suggesting that it was related to some dissociation mechanism. The stability at this pH of the CNBr preparation was also dependent on the enzyme concentration, whereas the glyoxyl derivative was much more stable than the soluble enzyme and exhibited the same stability in the concentrated or diluted form.

To elucidate if an effect of the stabilization of the multimeric structure could explain these results, the immobilized preparations were boiled in the presence of SDS and mercaptoethanol, and the supernatants thus obtained were submitted to SDS-PAGE (Fig. 5b). While some subunits of the enzyme immobilized on CNBr was desorbed from the support (lane 4), suggesting that not all the enzymes subunits were bound to the support, no protein could be extracted by this procedure from the glyoxyl preparations (lanes 5 and 6). This result suggested that glyoxyl agarose was able to attach the three subunits of the enzyme to the support; that is, immobilization was via a

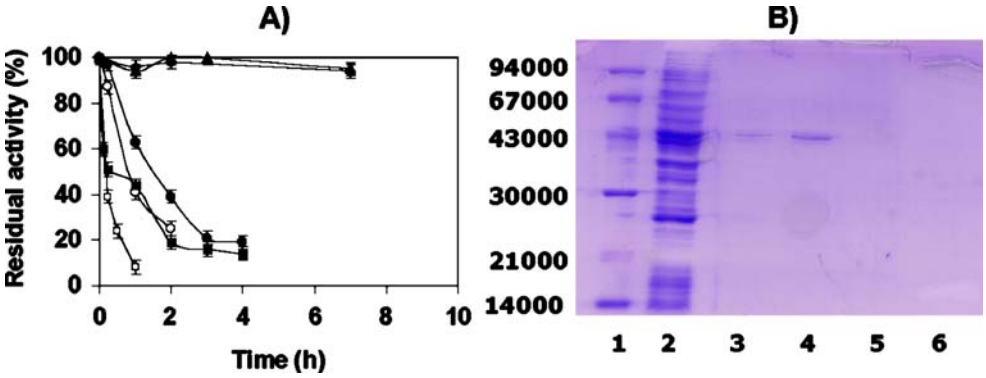

Fig. 5 **a** Inactivation courses of different preparations of glutamate dehydrogenase at pH 4. Experiments were carried out at 25°C in 100 mM sodium acetate. Symbols: *empty circles*, soluble GDH, 0.3 U/mL; *empty squares*, soluble GDH, 0.06 U/mL; *filled circles*, CNBr derivative, 0.3 U/mL; *filled squares*, CNBr derivative, 0.06 U/mL; *filled triangles,* glyoxyl agarose, 75 μmol/g 25°C 1.5 h 0.3 U/mL; *filled diamonds*, glyoxyl agarose, 75 μmol/g, 25°C, 1.5 h, 0.06 U/mL. **b** SDS-PAGE analysis of the proteins released from different GDH preparations. Lanes: *1*, molecular weight markers; *2*, crude extract GDH; *3*, purified GDH; *4*, CNBr preparation; *5*, glyoxyl agarose, 75 μmol/g, 25°C, 30 min; *6*, glyoxyl agarose, 75 μmol/g, 25°C, 1.5 h

plane that permits the simultaneous immobilization of all enzyme subunits. Thus, the enzyme stabilization achieved with highly activated glyoxyl agarose could be correlated not only with a multipoint covalent attachment of each individual subunit of the enzyme, but also with the involvement of each protein subunit in the enzyme immobilization, resulting in a stabilization of the quaternary structure of the enzyme (Fernández-Lafuente et al. 2001).

The optimal glyoxyl agarose derivative obtained is fully stable at pH 4 and 25°C, retaining more than 90% of its activity after incubation at 45°C for 24 h at pH 4 and more than 75% of the activity after the same period at 50°C, independently of the enzyme concentration employed.

Temperature–activity profile

The temperature–activity dependence profile of the enzyme showed a significant increment of the optimal temperature at pH 5 (around 15°C, see Fig. 6). The optimal temperature of glyoxyl agarose derivative is 75°C, whereas the soluble enzyme is fully inactive under these conditions. This should be consequence of the higher stability of the immobilized enzyme.

Determination of the activity of the immobilized enzyme under different experimental conditions with the different cofactors

The activity of the optimal-immobilized preparation enzyme was assayed using NAD^+, $NADP^+$ (using glutamate), and NADH (using α-ketoglutarate and NH_3; Fig. 7). The activity with NAD^+ was three- to fivefold higher than using $NADP^+$, with a maximum at pH 9. In the reduction reaction, using NADH, the activity was lower than in the other direction at pH 9 but was less dependent on the pH value, and more than 50% activity was found at pH 5 (in

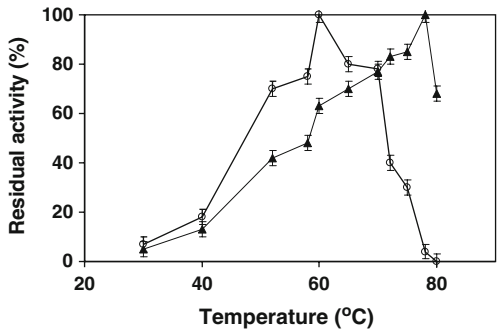

Fig. 6 Influence of temperature on the enzymatic activity of different GDH preparations. Activity assays were performed in 250 mM sodium glutamate in sodium acetate at pH 5 and different temperatures. The relative activity was calculated taking 100% the maximum observed activity as described in "Experimental section." Symbols: *empty circles*, soluble FDH; *filled triangles*, optimal glyoxyl agarose 6BCL preparation

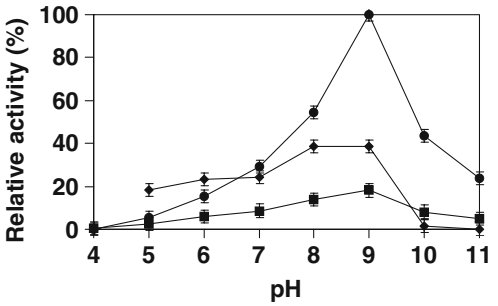

Fig. 7 Influence of pH on the enzymatic activity in both oxidation and reduction reactions of optimal glyoxyl agarose GDH preparation. Activity assays were performed at 66°C as described in "Experimental section." The relative activity was calculated taking 100% the maximum observed activity in glutamate oxidation with NAD^+. Symbols: *circles*, sodium glutamate oxidation, NAD^+; *diamonds*, ammonium α-ketoglutarate reduction, NADH; *squares*, glutamate oxidation, $NADP^+$

fact, at that pH value, activity was higher than using NAD^+ and glutamic acid).

Discussion

This paper described the cloning, purification, and immobilization of a very stable GDH. Having in mind that the *T. thermophilus* strains HB27 and HB8 present high sequence conservation among most of their genes, our results support the existence of two GDH activities in this species, which could correspond to the two genes encoding GDH homologues clustered in its chromosome. Thus, it is tempting to speculate that the NAD^+-dependent GDH could be more related with glutamate catabolism, while the enzyme described here could have also a role in glutamate synthesis in vivo. The enzyme has a trimeric structure, not very usual in other GDH.

The manuscript is an example that a proper immobilization technique may be used to improve the stability even of naturally stable enzymes, coupling the high natural stability of the enzyme to the stabilization obtained by multipoint and multisubunit immobilization.

This GDH is a nice example of a multimeric enzyme that under some conditions may be inactivated by the dissociation of their subunits. This GDH presents a very high stability even in their soluble state at neutral or alkaline pH values. However, at acidic pH values, the enzyme tends to dissociate resulting to enzyme inactivation, where the enzyme loses its thermopile character.

The multisubunit immobilization of this trimeric enzyme may be achieved in glyoxyl agarose, and this means that the enzyme recovers the thermophilic character even at an acidic pH value. Moreover, the results point that if each subunit is multipointly attached to the support, the stabilization may be improved. This may be deduced from the fact that while the multimeric structure is stabilized after 1 h, the stability reached may be improved if the immobilizaition conditions used favored the multipoint covalent attachment (highly activation of the support, long reaction times, and high temperatures).

Thus, this immobilized GDH presents very good prospects to be used as a biocatalyst or biosensor.

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