

Coimmobilization of L-asparaginase and glutamate dehydrogenase onto highly activated supports

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Abstract

In the present research work, production of coimmobilized derivatives of L-asparaginase and glutamate dehydrogenase was attempted. Comparison of immobilization of each enzyme independently with coimmobilization of the two enzymes unfolded important advantages of the latter, namely a decrease in the induction period (time before the maximum reaction rate is virtually achieved) and an increase in the maximum reaction rate. The effectiveness of the independent enzyme derivatives was low; however, it was enhanced by three-fold when the enzymes were coimmobilized onto the same agarose-glutaraldehyde support. Each supporting agarose bead may in fact be viewed as a nano-reactor with *in situ* reaction and separation (i.e. elimination of the ammonia formed), with the nanoenvironment surrounding each enzyme molecule being essentially devoid of steric hindrance. © 2001 Elsevier Science Inc. All rights reserved.

1. Introduction

L-asparaginase, an enzyme widely used on the clinical level as antitumoral agent, viz. in the treatment of acute leukaemia and lymphosarcoma [1–13], is obtained commercially from *Acinetobacter glutaminasificans*, *Pseudomonas* spp., *Escherichia coli* or *Erwinia chrysanthemi*; it hydrolyzes L-asparagine into L-aspartic acid and ammonia, a reaction that is essentially irreversible under physiological conditions [10]. Said enzyme is effective against neoplasias that require asparagine and obtain it from circulating plasma pools [14,15], presumably because cancer cells have diminished expression of asparagine synthetase [14]. Of all the aforementioned sources, L-asparaginase from *Escherichia coli* appears to be a suitable option; it has indeed been comprehensively characterized by chemical and physico-chemical methods [10,14,15]. In structural terms, its active form is a tetramer [14] of identical subunits [3], each with a molecular weight of 35.6 kDa.

Mammalian (bovine liver) glutamate dehydrogenase catalyzes the reversible oxidative deamidation of glutamate to α -ketoglutarate and ammonia, using either NAD⁺ or NADH as coenzyme (with comparable efficacies) [16]. Said enzyme is a hexamer of identical subunits, with a total molecular weight of 336 kDa. The role *in vivo* of glutamate dehydrogenase remains controversial: it is thought that it is used in the catalytic step underlying storage of nitrogen in the form of glutamate, which takes place when ammonia levels, at the cell level, are high. Should ammonia levels be low, glutamate dehydrogenase is alternatively used to degrade glutamate to ammonia and α -ketoglutarate [16]. The regulation of glutamate levels has a unique importance in the brain, since glutamate is a potent neurotransmitter.

There are three general reasons why immobilized enzymes are desirable, particularly in bioengineering: (i) the enzyme is retained in (or on) the beads (and hence in the bioreactor), which circumvents the need for upstream enzyme makeup and prevents downstream contamination of the product; (ii) immobilized enzymes keep their activity for a longer period than those in solution, because the natural flexibility of the proteinaceous backbone, essential for thermal unimolecular denaturation to take place, is restricted;

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and (iii) enzymes in immobilized form may be deliberately fixed in the vicinity of other enzymes that participate in a given reaction sequence, thereby increasing the overall catalytic efficiency of multistep conversions, because the entropic contribution, arising from the requirement of productive collision of the product(s) of the first reaction with the enzyme catalyzing the second reaction, is dramatically reduced when the reaction becomes intraparticle rather than interparticle [17]. Hence, if two enzymes acting in series are immobilized side by side on a given support matrix, the intermediate product (in our case, ammonia obtained from L-asparagine) has an extremely high probability of reacting further (to produce glutamate) before it can diffuse out of the bead [18].

The current (intrusive) therapy makes use of endovenous administrations of enzymes in soluble form, but is effective only to a certain degree because of two major shortcomings: L-asparaginase circulates in the blood system for only a short time before being taken up and broken down by native proteases; and its presence triggers adverse immunological side-effects, owing to the presence of such alien protein inside the human body, the severity of which may range from mild allergenic reaction to anaphylactic shock.

The development of strategies that will eventually permit structural and functional stabilization of both L-asparaginase and glutamate dehydrogenase via coimmobilization onto highly activated supports may increase the biomedical applicability of these enzymes. In fact, the enzyme-mediated hydrolysis of L-asparagine produces ammonia, which becomes a poison to the human kidney if present in high level; such risk can be easily overcome if another enzyme, possessing a strong preference for ammonia, is coimmobilized together with the former. These coimmobilized derivatives could then be utilized in extracorporeal devices to efficiently eliminate asparagine from the plasma [19,20], without the extra risks incurred in by bulk accumulation of ammonia.

Agarose beads were selected as suitable supports for coimmobilization, since they consist of a biocompatible three-dimensional network of highly hydrophilic and inert fibers; their surface is essentially covered by hydroxyl groups, which can easily be activated for covalent, multi-subunit immobilization of enzymes [21–29]. Attachment of the enzyme molecules to the support via short spacer arms, while providing intense enzyme-support interactions, allows full functional stabilization of their multimeric structure because (i) they are prevented from interacting with inactivating microenvironments, (ii) they are protected from the first steps of inactivation, viz. dissociation of subunits and conformational changes, (iii) they are protected from irreversible inactivation through aggregation, (iv) they become much more rigid, so thermal energy-driven vibrations and rotations of hydrogen and disulfide bonds are kept (at most) to a very low level, therefore protecting the enzyme against thermal denaturation, and (v) they are protected against proteolytic attack and action by denaturing (re)agent(s).

2. Materials and methods

2.1. Materials

2.1a. Enzymes. The enzymes used were L-asparaginase (type II) from *Escherichia coli*, purchased in a 50% (v/v) glycerol suspension from Fluka (Steinheim, Germany), and glutamate dehydrogenase from bovine liver, purchased in a 50% (v/v) glycerol suspension from Boehringer Mannheim (Mannheim, Germany).

2.1b. Chemicals. Agarose beads (4% cross-linked) were obtained from HISPANAGAR (Burgos, Spain). Glycidol (2,3-epoxy-1-propanol), L-asparagine monohydrate, β -NADH, boric acid, ethylenediamine, formaldehyde, TRIZMA® Hydrochloride, Schiff's reagent (fuchsin-sulfate), sodium acetate trihydrate and sodium borohydride were all purchased from Sigma (St. Louis, MO, U.S.A.). α -Ketoglutarate, in disodium salt form, was purchased from Boehringer Mannheim (Darmstadt, Germany). Sodium metaperiodate was purchased from Merck (Darmstadt, Germany). Sodium hydroxide, sodium hydrogen phosphate (monohydrate) and glycerol (87% (v/v) in water) were all purchased from Panreac Química (Barcelona, Spain). Glutaraldehyde (25% (v/v) in water) was purchased from Fluka. Tap water was purified in a Milli-Q Plus 185 system (Molsheim, France) to a final conductivity of ca. $18.2 \text{ M}\Omega \cdot \text{cm}^{-1}$.

2.1c. Analytical equipment. All spectrophotometric readings were carried out at 25°C using quartz cuvettes, in a UVIKON 930 UV-VIS spectrophotometer coupled with a KP91-00580 magnetic stirrer unit for the cuvette (Kontron Instruments, Madrid, Spain).

3. Experimental procedures

3.1. Activity assay for L-asparaginase

The activity assay developed for L-asparaginase is based on the production of ammonia during hydrolysis of L-asparagine, which is then degraded by glutamate dehydrogenase with concomitant oxidation of β -NADH; depletion of β -NADH is then monitored spectrophotometrically at 340 nm. Improved assay conditions comprise the addition, to a quartz cuvette containing a magnetic rod, of the following sequence of reactants: 2 mL of an aqueous solution 50 mM in NaH_2PO_4 (pH 7), 1.1 mM in L-asparagine and 0.11 mM in α -ketoglutarate; 25 μL of an aqueous solution 50 mM in NaH_2PO_4 (pH 7) and 43.4 mM in β -NADH; and 50 μL of an aqueous solution 50 mM in NaH_2PO_4 (pH 7), 50% (v/v) in glycerol and 8.81 μM in glutamate dehydrogenase. The mixture in the cuvette, thermostatted at 25°C, was gently stirred for ca. 3 min. The activity assay was initiated by addition into the cuvette of an aliquot of 100 μL of the L-asparaginase solution which is to be tested for

activity (typically with a protein concentration of 3.86×10^{-8} M); immediately thereafter, absorbance at 340 nm was read up to 10 min. Since the activity of L-asparaginase is directly proportional to the rate of production of ammonia, and since the large excess of glutamate dehydrogenase degrades it totally with concomitant oxidation of β -NADH, the (negative) slope of the linear portion of the plot of absorbance versus reaction time provides a measure of the activity of L-asparaginase.

3.2. Activity assay for glutamate dehydrogenase

The activity assay for glutamate dehydrogenase is based on the degradation of ammonia, and concomitant oxidation of β -NADH. Since there is only one enzyme involved in this assay, there is no need for it to proceed rapidly, so a relatively small amount of glutamate dehydrogenase can be used. This enzymatic activity assay is also based on the depletion of β -NADH, monitored at 340 nm. The assay, after careful tuning, comprises the sequential addition, to a quartz cuvette containing a magnetic rod, of the following reactants: 2 mL of an aqueous solution 50 mM in NaH_2PO_4 (pH 7), 1.1 mM in NH_4Cl and 0.11 mM in α -ketoglutarate; and 50 μL of an aqueous solution 50 mM in NaH_2PO_4 (pH 7) and 21.7 mM in β -NADH. The mixture in the cuvette, under magnetic stirring, is thermostatted at 25°C for ca. 3 min, and the activity assay is initiated by addition into the cuvette of an aliquot of 200 μL of the glutamate dehydrogenase solution, which is to be tested for activity (typically with a protein concentration of 2.20×10^{-7} M). Immediately after addition of the enzyme solution, absorbance at 340 nm was read during up to 10 min. Since the activity of glutamate dehydrogenase is directly proportional to the rate of consumption of ammonia, and therefore to the rate of disappearance of β -NADH, the negative slope of the linear part of the plot of absorbance versus reaction time provides a measure of the activity of glutamate dehydrogenase.

3.3. Activity assay for coacting L-asparaginase and glutamate dehydrogenase

The activity assay for the solutions containing both enzymes was again based on the degradation of ammonia, and subsequent oxidation of β -NADH. The optimized bienzymatic activity assay is thus based on the rate of disappearance of β -NADH, monitored at 340 nm. The carefully tuned assay comprises the addition, to a quartz cuvette containing a magnetic rod, of 2 mL of an aqueous solution 50 mM in NaH_2PO_4 , 1.1 mM in L-asparagine, 0.11 mM in α -ketoglutarate and 0.5 mM in β -NADH. The mixture in the cuvette, under magnetic stirring, is thermostatted at 25°C for ca. 3 min, and the activity assay is initiated by addition into the cuvette of an aliquot of 200 μL of the bienzymatic solution, which is to be tested for activity. Immediately after addition of this latter solution, absorbance at 340 nm was read during up to 10 min.

3.4. Preparation of supports for immobilization

3.4a. Glyoxyl-agarose supports. The procedure described by Guisán (1988) [21] was followed, with slight modifications: 105 g of agarose beads (cross-linked to 4%, and previously washed with plenty of deionized water) were weighed into a plastic flask, and water was added to a final volume of 180 mL (0.7 g of agarose is roughly equivalent to 1 mL). Following mild homogenization, 50 mL of 1.7 M NaOH containing 1.425 g NaBH_4 (as reducing agent) was slowly added. The flask was then placed in an ice bath, and glycidol was added dropwise (because it promotes a highly exothermic reaction) to a final concentration of 2 M. The mixture was then paddle-agitated at room temperature (ca. 25°C) for 15–18 h, and the gel was finally washed with excess deionized water. The activated gel (i.e. 150 mL_{gel}) thus obtained was added with 1 mL of aqueous 100 mM NaIO_4 per mL of gel (so as to reach 100 $\mu\text{mol}_{\text{aldehyde groups}}/\text{mL}_{\text{gel}}$). The gel was then suspended (1:10) in water to a final volume of 1500 mL, and oxidation was allowed to proceed for 1.5–2 h with paddle agitation; then, the gel was washed with plenty of deionized water. Confirmation of the presence of aldehyde groups in the gel was by addition of a few mg of (oxidized) gel to ca. 200 μL of Schiff's reagent in an eppendorf, which produced the expected pinky-purple coloration.

3.4b. Agarose-glutaraldehyde supports. The procedure described by Guisán (1988) [21] was again followed, with slight modifications: 70 g of glyoxyl-agarose gel (prepared according to the procedure described above) was weighed into a plastic beaker, and further added with 400 mL of 2 M ethylenediamine (pH 10). Following mild paddle agitation for ca. 2 h, 4 g of NaBH_4 was added and the paddle agitation was provided for an extra 2 h. The amine gel thus obtained was then sequentially washed with 1000 mL of 0.1 M sodium acetate (pH 4) (to eliminate sodium borohydride), 1000 mL of 0.1 M boric acid (pH 9) (to reduce electrostatic interactions and eliminate excess ethylenediamine), and finally plenty of deionized water. To 5 g of amine gel, 5.6 mL of 200 mM NaH_2PO_4 was added, and the pH of the resulting mixture was adjusted to 7 using diluted NaOH; 8.4 mL of aqueous 25% (v/v) glutaraldehyde was then added and the pH adjusted once again to 7. The mixture was subsequently paddle-agitated overnight, and the final gel was washed with excess deionized water.

3.5. Immobilization protocols

3.5a. Preparation of immobilized L-asparaginase. The preparation of these immobilized derivatives followed the procedure described by Balcão *et al.* (2001), [29] and encompassed addition of 16 mL aqueous 87% (v/v) glycerol onto a plastic flask, followed by homogenization with 16 mL of 25 mM NaH_2PO_4 (pH 7). To the resulting solution, 229 μL of a 4.2 $\text{mg}_{\text{L-asparaginase}}/\text{mL}_{\text{glycerol}}$ suspension was

added. Following mild homogenization, 2 g of agarose-glutaraldehyde (after activation with $40 \mu\text{mol}_{\text{aldehyde groups}}/\text{mL}_{\text{gel}}$) was added, and the flask was stoppered tightly and placed in an orbital shaker at 4°C . Samples of both the suspension and the supernatant were regularly withdrawn and assayed for activity, together with the blank (which consisted of a 4 mL-sample of enzyme solution, withdrawn prior to addition of the support). The immobilization procedure was terminated after 45 min by simply washing the derivative with plenty of deionized water, resuspending it in aqueous 50 mM NaH_2PO_4 (pH 7) at 4°C , and storing the suspension in an ice bath.

3.5b. Preparation of immobilized glutamate dehydrogenase.

The preparation of these immobilized derivatives encompassed addition of 16 mL aqueous 87% (v/v) glycerol into a plastic flask, followed by homogenization with 16 mL of 25 mM NaH_2PO_4 (pH 7) at 4°C . To the resulting solution, 5200 μL of a $10 \text{ mg}_{\text{glutamate dehydrogenase}}/\text{mL}_{\text{glycerol}}$ suspension was added. Following mild homogenization, 2 g of agarose-glutaraldehyde (after activation with $40 \mu\text{mol}_{\text{aldehyde groups}}/\text{mL}_{\text{gel}}$) was added, and the flask was stoppered tightly and placed in an orbital shaker at 4°C . Samples of both the suspension and the supernatant were regularly withdrawn and assayed for activity, together with the blank (which consisted of a 4 mL-sample of enzyme solution, withdrawn prior to addition of the support). The immobilization procedure was terminated after 45 min by simply washing the derivative with plenty of deionized water, resuspending it in 50 mM NaH_2PO_4 (pH 7) at 4°C , and storing the suspension in an ice bath.

3.5c. Preparation of coimmobilized L-asparaginase and glutamate dehydrogenase. The preparation of these coimmobilized derivatives encompassed addition of 16 mL of aqueous 87% (v/v) glycerol into a plastic flask, followed by homogenization with 16 mL of 25 mM NaH_2PO_4 (pH 7). To the resulting solution, 229 μL of a $4.2 \text{ mg}_{\text{L-asparaginase}}/\text{mL}_{\text{glycerol}}$ suspension and 457 μL of a $10 \text{ mg}_{\text{glutamate dehydrogenase}}/\text{mL}_{\text{glycerol}}$ suspension were added to produce a 1:1 enzyme mixture of the resulting derivative. Following mild homogenization, 2 g of agarose-glutaraldehyde (after activation with $40 \mu\text{mol}_{\text{aldehyde groups}}/\text{mL}_{\text{gel}}$) was added, and the flask was stoppered tightly and placed in an orbital shaker at 4°C . Samples of both the suspension and the supernatant (i.e. bienzymatic solutions) were regularly withdrawn and assayed for activity, together with the blank (which consisted of a 4 mL-sample of solution containing both enzymes, withdrawn prior to addition of the support). After 30 min of contacting the bienzymatic solution with the support, the immobilization procedure was terminated by simply washing the derivative with plenty of deionized water; it was then resuspended in cold 50 mM NaH_2PO_4 (pH 7), and the resulting suspension was kept in an ice bath. This procedure was repeated when preparing other coimmobilized derivatives: using 229 μL of $4.2 \text{ mg}_{\text{L-asparaginase}}/\text{mL}_{\text{glycerol}}$ suspen-

sion and 2285 μL of $10 \text{ mg}_{\text{glutamate dehydrogenase}}/\text{mL}_{\text{glycerol}}$ suspension to produce a 1:5 enzyme mixture of L-asparaginase and glutamate dehydrogenase in the resulting derivative; using 58 μL of $4.2 \text{ mg}_{\text{L-asparaginase}}/\text{mL}_{\text{glycerol}}$ suspension and 2285 μL of $10 \text{ mg}_{\text{glutamate dehydrogenase}}/\text{mL}_{\text{glycerol}}$ suspension to produce a 1:20 enzyme mixture of L-asparaginase and glutamate dehydrogenase in the resulting derivative; or using 12 μL of $4.2 \text{ mg}_{\text{L-asparaginase}}/\text{mL}_{\text{glycerol}}$ suspension and 2285 μL of $10 \text{ mg}_{\text{glutamate dehydrogenase}}/\text{mL}_{\text{glycerol}}$ suspension to produce a 1:100 enzyme mixture of L-asparaginase and glutamate dehydrogenase in the resulting derivative.

3.6. Conversion of L-asparagine to glutamate

3.6a. Reactions using independent immobilized enzymes.

Since reduction of the independent derivatives after the immobilization procedure proved deleterious for the immobilized enzymes, both independent derivatives were immediately resuspended in cold phosphate buffer (50 mM, pH 7) and further kept in ice. The derivatives were not reduced with sodium borohydride, so the enzyme continued interacting with the support; however, the low temperature slowed down the reactional consequence of such interactions. Independent derivatives were resuspended using two dilution factors for L-asparaginase, viz. 1:10 (200 mg derivative + 2.9 mL of 50 mM NaH_2PO_4 , pH 7, at 4°C) and 1:20 (200 mg coimmobilized derivative + 5.8 mL of 50 mM NaH_2PO_4 , pH 7, at 4°C), and two dilution factors for glutamate dehydrogenase, viz. 1:10 (700 mg derivative + 10 mL of 50 mM NaH_2PO_4 , pH 7, 4°C) and 1:100 (700 mg coimmobilized derivative + 100 mL of 50 mM NaH_2PO_4 , pH 7, 4°C). Reactions were performed by employing variable amounts of both (independent) enzyme derivatives. The reaction vessel was a quartz cuvette containing a magnetic rod and thermostatted at 25°C ; the reaction medium encompassed 2 mL of an aqueous solution of 50 mM NaH_2PO_4 (pH 7), 1.1 mM L-asparagine, 0.11 mM α -ketoglutarate and 0.5 mM β -NADH. To the reaction medium, the designated amount of glutamate dehydrogenase-derivative suspension was added, and the reaction was initiated with the subsequent addition of the designated amount of L-asparaginase-derivative suspension. The *in situ* production of ammonia (following hydrolysis of L-asparagine by the L-asparaginase derivative) and its degradation by the glutamate dehydrogenase-derivative, with consequent oxidation (and hence depletion) of β -NADH, was monitored at 340 nm for ca. 20 min.

3.6b. Reactions using coimmobilized enzymes. Coimmobilized derivatives were resuspended according to two dilution factors, viz. 1:5 (100 mg coimmobilized derivative + 715 μL of 50 mM NaH_2PO_4 , pH 7, at 4°C) and 1:10 (100 mg coimmobilized derivative + 1430 μL of 50 mM NaH_2PO_4 , pH 7, at 4°C). The reaction vessel was again a quartz cuvette containing a magnetic rod thermostatted at

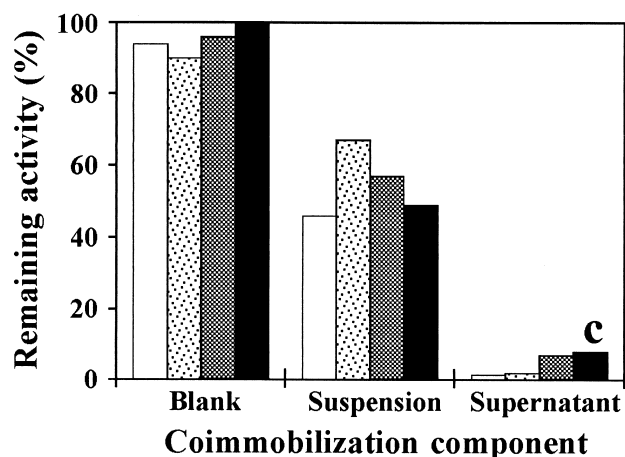
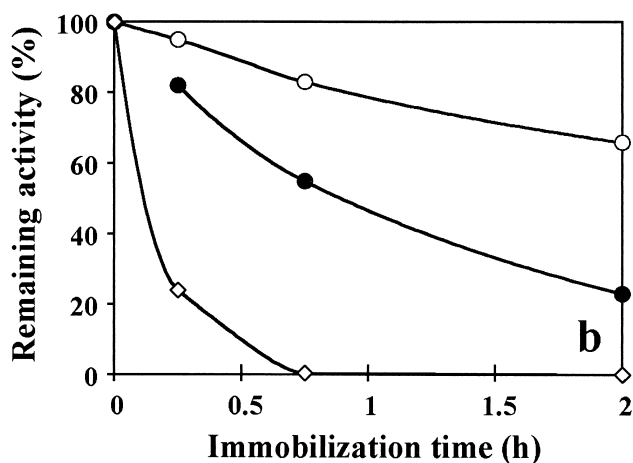
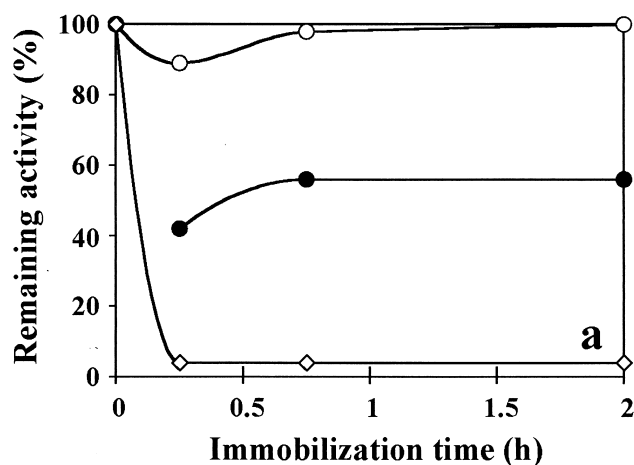


Fig. 1. Analysis of the remaining activity of L-asparaginase (a), glutamate dehydrogenase (b), and coacting L-asparaginase and glutamate dehydrogenase (c) during immobilization onto agarose-glutaraldehyde supports activated with $40 \mu\text{mol}_{\text{aldehyde groups}}/\text{mL}_{\text{gel}}$. Data points represent the percentage activity of the blank (\circ), the suspension (\bullet) and the supernatant (\diamond), for immobilization of independent enzymes, and 1:1 (\square), 1:5 (\square), 1:20 (\square) and 1:100 (\blacksquare) ratios of coimmobilized L-asparaginase:glutamate dehydrogenase.

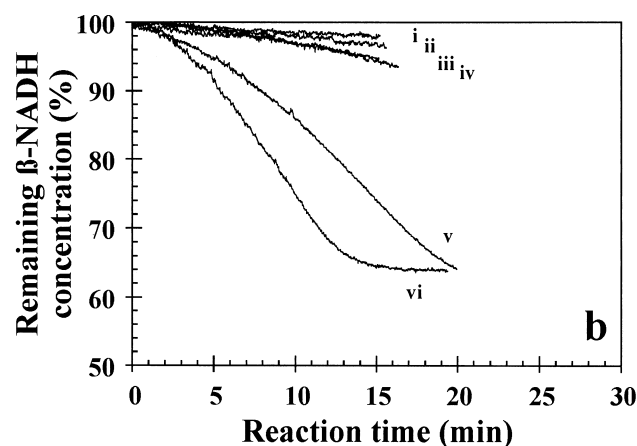
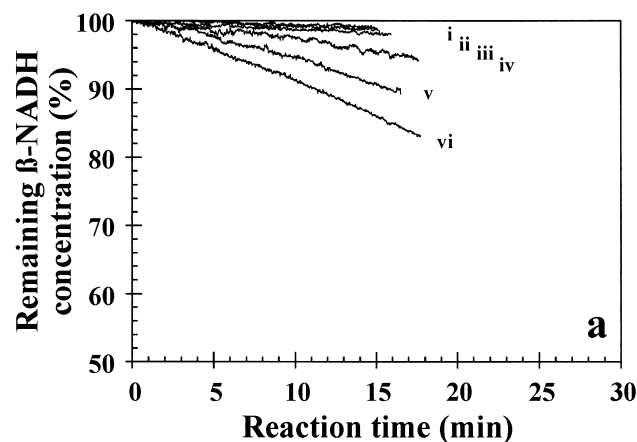


Fig. 2. Analysis of the remaining concentration of β -NADH during hydrolysis of L-asparagine with (a) $2.4 \mu\text{L}$ independent L-asparaginase gel and various amounts of independent glutamate dehydrogenase gel (viz. i: $0.5 \mu\text{L}$; ii: $1 \mu\text{L}$; iii: $2 \mu\text{L}$; iv: $5 \mu\text{L}$; v: $20 \mu\text{L}$; and vi: $40 \mu\text{L}$), and (b) $18 \mu\text{L}$ independent L-asparaginase gel and various amounts of independent glutamate dehydrogenase gel (viz. i: $0.5 \mu\text{L}$; ii: $1 \mu\text{L}$; iii: $2 \mu\text{L}$; iv: $5 \mu\text{L}$; v: $20 \mu\text{L}$; and vi: $40 \mu\text{L}$).

25°C ; the reaction medium encompassed 2 mL of an aqueous solution of $50 \text{ mM NaH}_2\text{PO}_4$ ($\text{pH } 7$), 1.1 mM L-asparagine, 0.11 mM α -ketoglutarate and 0.5 mM β -NADH. The reaction was initiated with addition of variable amounts of coimmobilized derivative suspension. The *in situ* production of ammonia (following hydrolysis of L-asparagine by the coimmobilized L-asparaginase) and its degradation by coimmobilized glutamate dehydrogenase, with consequent oxidation (and hence depletion) of β -NADH, was monitored at 340 nm for ca. 20 min .

4. Results and Discussion

4.0a. Independent enzyme immobilization. Agarose-based supports, pre-activated with glutaraldehyde, react preferentially with the terminal amino residues of proteins. When

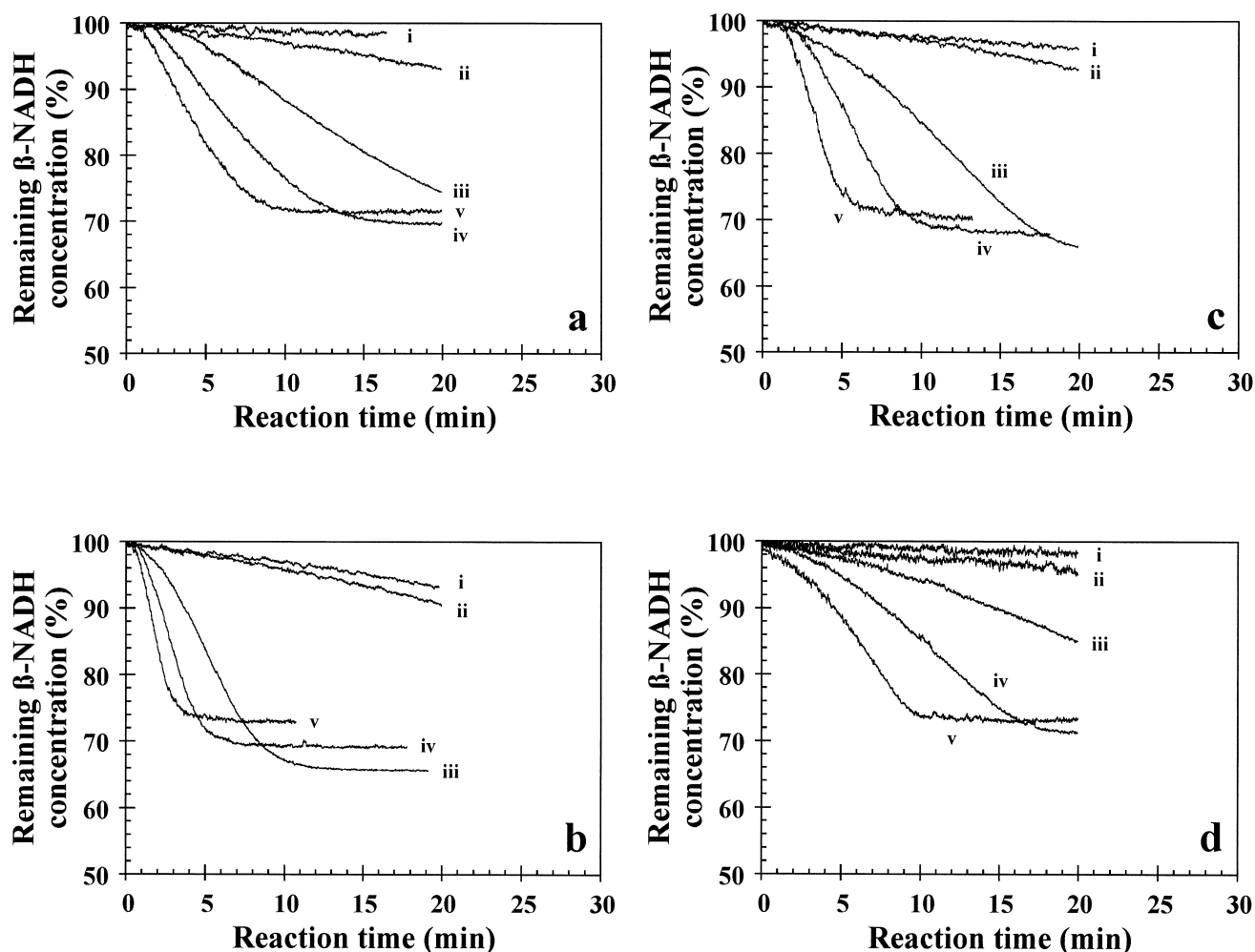


Fig. 3. Analysis of the remaining concentration of β -NADH during hydrolysis of L-asparagine with (a) various amounts of coimmobilized 1:1 gel (viz. i: 2.5 μ L; ii: 5 μ L; iii: 20 μ L; iv: 50 μ L; and v: 100 μ L), (b) various amounts of coimmobilized 1:5 gel (viz. i: 2.5 μ L; ii: 5 μ L; iii: 20 μ L; iv: 50 μ L; and v: 100 μ L), (c) various amounts of coimmobilized 1:20 gel (viz. i: 2.5 μ L; ii: 5 μ L; iii: 20 μ L; iv: 50 μ L; and v: 100 μ L), and (d) various amounts of coimmobilized 1:100 gel (viz. i: 2.5 μ L; ii: 5 μ L; iii: 20 μ L; iv: 50 μ L; and v: 100 μ L).

the activation degree is high, as was the case, and the enzyme is allowed to contact the support for a long time (say, above 2 h) in an environment set at low ionic strength, several bonds will be established between enzyme and support [27,30]; the establishment of new bonds between those two highly complex structures requires longer reaction times [27], possibly because of difficulties in aligning correctly the enzyme surface and the activated groups on the support surface. The activity of the free L-asparaginase solution (see Figure 1a) remained virtually constant during the timeframe of the immobilization trials. Furthermore, the percent activity retained by the derivative was 52%, whereas the blank exhibited its full initial activity. These results are in close agreement with those reported by Fernández-Lafuente *et al.* (1999), Blanco and Guisán (1989), and Blanco *et al.* (1989) [27,30,31].

Bearing in mind that glutamate dehydrogenase must be in great excess in the reaction medium, an independent derivative of this enzyme was prepared, but making a large

excess of enzyme available to the support. The results obtained during the timeframe of the immobilization procedure are displayed in Figure 1b. As can be observed, glutamate dehydrogenase is immobilized fast at low ionic strength. However, the suspension lose activity as time elapsed, probably because more bonds formed as more enzyme molecules became attached. This could promote a slight distortion in the three-dimensional architecture of the enzyme, thereby inactivating some of the enzyme molecules already immobilized. For this reason, the independent derivatives of glutamate dehydrogenase were further prepared simply by contacting the enzyme with the support for 45 min. This procedure led to glutamate dehydrogenase derivatives that retained more than 50% of the activity of the initial (free) enzyme solution (see Figure 1b).

4.0b. Enzyme coimmobilization. As can be realized from inspection of Figure 1c, the activity of the free enzyme solution (i.e. the supernatant) decays appreciably during the

Table 1

Maximum reaction rates (ϑ_{\max}) using distinct amounts of coimmobilized enzymes L-asparaginase (L-asnase) and glutamate dehydrogenase (GDH)

Amount of coimmobilized derivative	ϑ_{\max} ($\mu\text{mol}_{\text{NH}_4^+}/\text{min}$, at 25°C and pH 7)			
	Ratio of L-asnase: GDH			
	1:1	1:5	1:20	1:100
2.5 μL	0.0039	0.0105	0.0053	0.0017
5 μL	0.0071	0.0073	0.0081	0.0043
20 μL	0.0399	0.1139	0.0508	0.0153
50 μL	0.0700	0.1765	0.1001	0.0424
100 μL	0.1184	0.3119	0.1913	0.0835

time frame of the immobilization protocol, for all coimmobilization proportions. By the end of the coimmobilization timeframe, the percent activity of the enzyme derivative is ca. 45% (obtained as the difference between the activity of the suspension and that of the supernatant) for the 1:1 derivative, 65% for the 1:5 derivative, 50% for the 1:20 derivative and 41% for the 1:100 derivative, whereas the blank exhibited ca. 94% (1:1), 90% (1:5), 96% (1:20) and 100% (1:100), respectively, of the initial activity (see Figure 1c); this implies that the coimmobilized derivatives retained ca. 42% (for the 1:1 derivative), 59% (for the 1:5), 48% (for the 1:20) and 41% (for the 1:100) of the initial activity of the bienzymatic solution.

When performing this type of enzyme immobilization, it is usually necessary to reduce the derivative upon completion of the immobilization procedure; this will convert the non-reacted groups on the surface of the support into inert hydroxyl groups, which ensures that the immobilized enzyme molecules will not interact further with such groups. In our case, however, the coimmobilized derivatives lost all activity after reduction with sodium borohydride and washing with deionized water (results not shown). At first glance, two possible explanations appear plausible: reduction of the coimmobilized derivatives may have inactivated one (or both) enzymes, or one of the enzymes might have adsorbed competitively with the other enzyme that was already immobilized, thus allowing it to be bleached away during

Table 2

Maximum reaction rates (ϑ_{\max}) using distinct amounts of independent immobilized enzymes L-asparaginase (L-asnase) and glutamate dehydrogenase (GDH)

Amount of L-asnase derivative	Amount of GDH derivative	ϑ_{\max} ($\mu\text{mol}_{\text{NH}_4^+}/\text{min}$, at 25°C and pH 7)
2.4 μL	0.5 μL	0.0027
	1 μL	0.0042
	2 μL	0.0031
	5 μL	0.0059
	20 μL	0.0147
18 μL	40 μL	0.0202
	0.5 μL	0.0035
	1 μL	0.0048
	2 μL	0.0060
	5 μL	0.0090
	20 μL	0.0408
	40 μL	0.0708

washing; this second hypothesis seems unlikely because it would be necessary that all enzyme molecules of one kind would be exchanged in the adsorption sites of all enzyme molecules of the other kind. Following the first, more probable hypothesis, it was decided not to reduce the coimmobilized derivatives upon completion of the immobilization procedure; instead, one resuspended them in buffer, at 4°C, followed by storage in ice (as the low temperature will slow down further interactions between attached enzyme molecules and non-reacted groups on the surface of the support) before performing the experiments.

4.0c. Performance of independent immobilized enzymes.

The results, in the form of remaining β -NADH concentration, obtained for the batch hydrolysis of L-asparagine using independent derivatives of L-asparaginase and glutamate dehydrogenase, are displayed in Figure 2a for 2.4 μL of the L-asparaginase derivative and variable amounts of the glutamate dehydrogenase derivative, and in Figure 2b for 18 μL of the L-asparaginase derivative and the same variable amounts of the glutamate dehydrogenase derivative.

Table 3

Maximum reaction rates ($\mu\text{mol}_{\text{NH}_4^+}/\text{min}$, at 25°C and pH 7) catalyzed by L-asparaginase (L-asnase) and glutamate dehydrogenase (GDH) for various L-asparagine concentrations

Proportion of L-asnase:GDH in coimmobilized derivative	Amount of coimmobilized derivative used to perform reaction	Concentration of L-asparagine (mM)		
		0.55	1.1	2.2
1:1	50 μL	0.0015	0.0700	0.0009
	100 μL	0.0048	0.1184	0.0021
1:5	50 μL	0.0153	0.1765	0.0203
	100 μL	0.0399	0.3119	0.0509
1:20	50 μL	0.0329	0.1001	0.0548
	100 μL	0.0658	0.1913	0.1072
1:100	50 μL	0.0541	0.0424	0.0573
	100 μL	0.0908	0.0835	0.1012

Note: The reaction mixture encompassed 2 mL of a 50 mM (pH 7) NaH_2PO_4 solution, that is 0.11 mM in α -ketoglutarate and 0.5 mM in β -NADH.

4.0d. Performance of coimmobilized enzymes. The results, in the form of remaining β -NADH concentration, obtained for the batch hydrolysis of L-asparagine using coimmobilized derivatives of L-asparaginase and glutamate dehydrogenase, are displayed in Figure 3a for the 1:1 proportion of the former to the latter enzyme, in Figure 3b for the 1:5 proportion, in Figure 3c for the 1:20 proportion, and in Figure 3d for the 1:100 proportion.

4.0e. Separate immobilization versus coimmobilization. When comparing immobilization versus coimmobilization in different supports, important differences may be noticed, viz. in the induction period (i.e. the time for attainment of the maximum reaction rate) of ammonia production and also in the maximum reaction rate. Adding increasing amounts of independent glutamate dehydrogenase derivative to a fixed (small) amount of independent L-asparaginase derivative (see Figure 2a) had virtually no effect on the aforementioned induction period; however, when the amount of L-asparaginase was increased 7.5-fold, significant differences in said induction period arose (see Figure 2b). Nevertheless, the efficiency of the independent enzyme derivatives was quite small. When the enzymes were coimmobilized onto the same agarose-glutaraldehyde support, their efficiency became three-fold higher (see Figures 3a, b, c, d).

As can be observed from inspection of Table 1, the rate of reaction becomes essentially independent of the concentration of L-asparagine when the proportion of glutamate dehydrogenase in the coimmobilized derivative is 100-fold that of L-asparaginase; the induction periods necessary for ammonia production are also drastically reduced. The concentration of L-asparagine in the reaction medium seems to have an important effect in the rate of reaction (see Table 3); glutamate dehydrogenase is somehow inhibited at higher concentrations of L-asparagine (see Table 3), because the maximum reaction rate is always achieved at a concentration of L-asparagine of 1.1 mM.

The results obtained in the coimmobilization experiments suggest that each agarose bead containing both enzymes coimmobilized can be viewed as an integrated nano-reactor, with *in situ* reaction and separation of products (via elimination of ammonia). By using agarose (cross-linked to 4%) activated with glutaraldehyde as support for coimmobilization, the (nano)environment surrounding each molecule of enzyme is virtually devoid of steric hindrance. When separate derivatives were utilized to perform that reaction, the ammonia produced inside the pores of the L-asparaginase derivatives needs to diffuse out of the agarose beads into the reaction medium, and diffuse back into the agarose beads containing immobilized glutamate dehydrogenase. However, when both enzymes were coimmobilized onto the same support, this sequence of steps was drastically reduced, as the ammonia produced inside the pores by the first immobilized enzyme becomes immediately accessible to the second immobilized enzyme. Two immediate advan-

tages of this (entropically favored) approach are (i) the shortening of the induction periods and (ii) the enhancement of the effectiveness of the immobilized particles, with concomitant increase in the maximum reaction rate.

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