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Catalytic activity of immobilized fumarase

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Abstract

In order to evaluate the influence of the immobilization techniques of fumarase on its immobilization yield and on its catalytic activity, it was covalently bonded to the surface of polymers (consisting of suitably functionalized ethylene–vinyl alcohol copolymers and functionalized poly(acrylamides), and physically entrapped into cross-linked poly(acrylamide) gels.

The kinetic parameters of the hydration reaction of fumarate to L-malate were obtained by determining the UV absorbance variation of the fumarate double bond at 290 nm.

When the enzyme is covalently bonded, both activity and stability of the enzymatic preparations are low; however, when fumarase is bonded to ethylene–vinyl alcohol copolymers by less denaturating and more spacing coupling agents (as glutaraldehyde), a better residual enzyme activity was obtained, and it was seen that this latter depended on the amount of bonded enzyme. Also the influence of the hydrophilicity of the polymer matrix on the amount of bonded enzyme and on its activity was evaluated.

Satisfactory results were obtained by physical entrapment of the enzyme into poly(acrylamide) gel, with quantitative immobilization yields, a rather good enzyme activity ($\eta = 38 \pm 5\%$), and a constant catalytic activity, under operative conditions, for several days.

The inhibiting effect of methanol concentrations up to 20% (v/v), in the reaction medium, was also evaluated for the different immobilized enzyme preparations.

Keywords: Immobilized fumarase; Entrapment; Covalent bond; Poly(acrylamide) gel

1. Introduction

Fumarase is a carbon–oxygen hydrolyase catalyzing, in the Krebs cycle, the reversible and stereospecific hydration of fumarate to L-malate.

The native enzyme (having a molecular weight of 194,000 Da), consists of four tetrameric units linked together by non-covalent bonds among four identical subunits. Each one of these subunits contains three free thiol groups, in the absence of disulfide bridges [1–4].

Since some reagents able to interact with the free thiol groups can induce the dissociation of the enzyme into its subunits, bringing about a consequent loss of activity, it is reasonable to guess that these groups play an important role in the stabilization of the tetrameric structure of the active enzyme.

Since by chemical hydration of fumaric acid a racemic mixture of D,L-malic acid is obtained, fumarase is industrially employed for the production of L-malic acid generally employing physically entrapped microbial cells containing high amount of the enzyme [5]. This biochemical reaction is carried out

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in a water solution of fumarate, with 80% conversion to L-malate. The reaction is remarkably affected by reaction conditions such as ionic strength, pH, solvent dielectric constant and polarity, possibility of hydrogen bond formation and so on [6–8].

In a previous work, we had observed that the nature of the reaction medium could modify both kinetic and thermodynamic constants of the reaction; the presence of an organic solvent in the reaction medium, for instance, decreases the enzymatic catalytic activity, probably due to protein denaturation and consequent (often irreversible) loss of activity [9].

The employment as biocatalyst of immobilized whole cells offers, with respect to the use of specific purified and immobilized enzymes, interesting advantages of simplicity and economy, coupled to drawbacks like a lower reaction rate because of higher diffusion resistance due to the presence, besides the polymer membrane used for the immobilization, also of the bacterial wall. Moreover, the employment of cells containing their whole enzymic and co-enzymic endowment can make possible reactions of catabolic type further on transforming the desired reaction products.

The immobilization of purified fumarase, due to its complex structure, can however, let rise problems of deactivation, particularly if bifunctional chemical reagents are employed.

In this paper we describe the immobilization of fumarase to different polymer matrices, and the evaluation of its enzymatic activity with time, in comparison with that of the free enzyme, both in water solution and in the presence of methanol. Methanol was chosen as organic co-solvent since, as previously observed, its denaturing action on the enzyme is often reversible [10].

In order to evaluate the influence of the immobilization system on the amount and activity of bonded enzyme, this latter was both covalently grafted to various polymer surfaces and physically entrapped in a poly(acrylamide) gel.

2. Materials and methods

2.1. Chemicals

Fumarase from pig heart (as crystalline suspension in 3.2 M $(NH_4)_2SO_4$, 0.05 M KH_2PO_4, 0.014 M 2-mercaptoethanol, pH = 7.5, declared activity = 400 U/mg protein, Sigma) was used. The substrate was neutral sodium fumarate, purity > 99% (Fluka). Other reagents: formamide (FA, Fluka) distilled at 10^{-2} mbar; triethylamine (TEA, CARLO ERBA) and *n*-hexane (CARLO ERBA) distilled at normal pressure.

As first polymer support we employed an ethylene–vinyl alcohol copolymer (EVAL, DAJAK) containing ethylene and vinyl alcohol units in a 40/60 molar ratio), provided with good mechanical and physico-chemical properties. Its hydroxy groups were employed for its functionalization, i.e. the introduction of carboxy and aldehydic derivatives, as described in Fig. 1.

As second matrix we employed commercial Enzacryl polymers both of AA type (*p*-aminophenyl poly(acrylamide), Fluka), and AH (poly(acrylhydrazide), Fluka).

As for the physical entrapment into poly(acrylamide) gel, the enzyme immobilization takes place

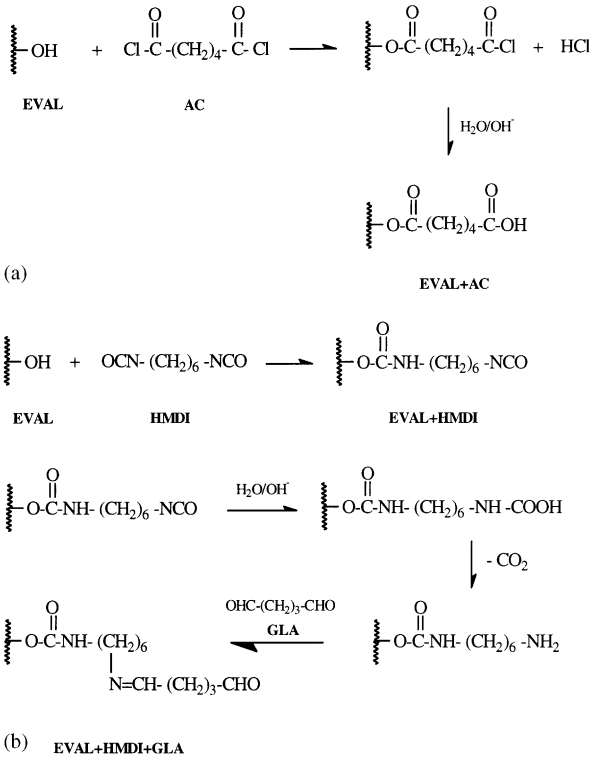

Fig. 1. Reaction scheme for synthesis of EVAL derivatives: introduction of carboxy (a); aldehydic groups (b).

simultaneously with the formation of the cross-linked insoluble polymer formed by co-polymerization between acrylamide and bis-acrylamide.

In order to maximize the surface/volume ratio, polymer particles having an average diameter of 100 μm were employed.

2.2. Immobilization reactions

2.2.1. Covalent bonding onto functionalized EVAL

The esterification reaction of EVAL by 0.1N solution of adipoyl chloride (AC, Fluka) in anhydrous formamide, in an amount sufficient to cover the polymer sample, was carried out for 3 h at 50°C and then for 21 h at room temperature. The HCl formed in the reaction was neutralized with an equivalent amount of anhydrous triethylamine.

After reaction, the polymer particles were washed several times with distilled water, and the residual –COCl groups hydrolized to carboxy groups, by adding 0.5 N NaOH for 3 h at room temperature. After further washing with distilled water, the carboxy groups were activated by reaction with a 10% (w/v) solution of *N*-(3-dimethylaminopropyl)-*N*′-ethyl carbodiimide hydrochloride (EDC, Fluka) in 0.2 M MES buffer (morpholinethanesulphonic acid, Fluka) at 0°C for 3 h. The powder was finally washed with MES buffer and dried under vacuum at 30°C for 2 days.

The successive reaction with the enzyme solution was effected using two different fumarase concentrations (1.7 and 15.3 U/ml) at 25°C, for 24 h under gentle stirring. The solution was then removed and the polymer washed with phosphate buffer (PBS) for 24 h at room temperature.

EVAL was also derivatized with hexamethylene diisocyanate (HMDI, Fluka) as 35% (v/v) solution in anhydrous *n*-hexane. The reaction was carried out at 50–60°C for 24 h under nitrogen flow, in the presence of a catalyst consisting of small amounts of TEA and dibutyltin dilaurate (in the 1:4 ratio).

The suspension is then filtered, the polymer washed with *n*-hexane and dried. The free isocyanate groups of the functionalized EVAL are then hydrolized to NH_2 groups by reaction with 0.025N NaOH solution at room temperature for 48 h. After washing with distilled water, glutaraldehyde (1% (v/v) water solution, GLA, Fluka) at pH 4 is added, and allowed to react for 24 h at room temperature. The powder is finally carefully washed with water and dried under vacuum at 30°C for 2 days.

In this case, the enzyme immobilization was carried out at different fumarase concentrations and a reaction time of 4 h was employed. The subsequent washing of the powders was made as previously described.

2.2.2. Covalent bonding onto Enzacryl AA and AH

Both supports were functionalized by reaction with a 10% (w/v) aqueous solution of $NaNO_2$ and 2 N HCl (pH $\cong$ 2) for 30′ at 0°C. The suspension was then centrifuged and the obtained powder was neutralized with 0.05 M PBS (pH = 7.3), again centrifuged and used for the enzyme immobilization, carried out for 4 h at room temperature under gentle stirring. The polymer–enzyme preparation was then washed for 24 h with PBS.

2.2.3. Entrapment in poly(acrylamide) gel

The enzyme entrapment takes place simultaneously with formation of the polymer gel, 3 ml of a PBS enzyme solution (from 3 to 10 U/ml) were added, under nitrogen flow, to PBS solutions (11 ml) of acrylamide (Fluka) and *N*,*N*′-methylene-bis-acrylamide (Fluka, concentration of 7.23 and 0.37% (w/v), respectively). As polymerization initiators 500 μl of a 3% solution of *N*,*N*,*N*′,*N*′-tetramethylene diamine and 500 μl of a 1.5% solution of ammonium peroxodisulphate. The total solution volume was always 15 ml.

The reaction was performed for 5′ at 0°C and then for 45′ in a cool water bath. The polymer–enzyme insoluble preparation was then washed with PBS in the usual way.

2.3. Kinetics of the enzyme reactions

The kinetic parameters of the reversible hydration reaction of fumarate to L-malate catalyzed by both free and immobilized fumarase have been determined by absorbance variations of the fumaric acid double bond, at 290 nm, employing a double-beam HITACHI U-2000 spectrophotometer. The measurements were made at 25°C, in 0.05 M PBS, pH = 7.3 as previously described for free fumarase [9].

Kinetic measurements with the immobilized enzyme were carried out by adding, in a stirred vessel thermostated at 25°C, the substrate solution to a known amount of immobilized enzyme, and measur-

ing at time intervals in the order of about 10″ the absorbance of samples of the reaction solution.

When the enzyme–polymer preparation consisted of poly(acrylamide) gel, this latter was previously cut in small pieces, in order to increase its surface/volume ratio, and to minimize consequently diffusion limitations for reagents and reaction products.

The parameters determined for the enzyme immobilized both covalently and physically were: the immobilization yield (Y, obtained by the ratio between the bonded enzyme units and those initially contained in the enzyme solution used for the bonding reaction) and the performance ratio η, i.e. the ratio between the activity of the immobilized enzyme and that of the same amount of free enzyme, in water or buffer solution.

The activity constancy with time of the immobilized preparations were also evaluated, by repeating the η measurements after time intervals of 2–3 days, under the usual reaction conditions, employing the same enzyme preparations stored in PBS at 4°C.

All the preparations containing the immobilized enzyme were also tested in the presence of methanol in the reaction medium, by evaluating their performance ratio (η) in 0.05 M PBS at pH = 7.3, with 0.04 M fumarate solutions. The residual activity of the immobilized enzyme was firstly determined in PBS containing 10 or 20% (v/v) of CH_3OH, and then again in methanol-free PBS, aiming at checking the reversibility of the possible inhibition. In all cases methanol contacted the enzyme only for the time necessary for the kinetic tests.

3. Results and discussion

Before investigating the behavior of the immobilized fumarase, some kinetic parameters relevant to the free enzyme operating in an aqueous medium have been determined.

The initial rate measurements of the dehydration reaction of L-malate, calculated for different substrate concentrations, were repeated at different fumarase concentrations. From these experimental data it was possible to calculate both V_{max} and K_m, while from the relation $V_{max} = k_{cat}[E]$ a value of $k_{cat} = 0.0062$ μM/s/U was determined.

It was so determined that, since one fumarase unit transforms 0.372 μmol/min of L-malate into fumarate, the specific enzyme acivity is 148.8 U/mg instead of 400 U/mg (as declared by Sigma).

In Table 1 the so obtained experimental values of K_m and V_{max}, for the hydration reaction of fumarate and for the dehydration of malate, are reported.

The new values obtained for the catalytic constants (0.0168 and 0.0424 μM/s/U for the dehydration and the hydration reaction, respectively) have confirmed a higher activity of fumarase for the direct hydration of fumarate. Moreover, the K_m values for both reactions increased with the enzyme concentration, showing a pattern of competitive inhibition. In fact, from Fig. 2, where the V_{max}/K_m ratio versus $[E]$ is reported, we can observe that the slope of the curve is lower for the reverse reaction (compared to the direct one), because of a higher K_m increase and a lower V_{max} increase (Table 1). The inhibition of both enzymic reactions is probably due to the presence of L-malate, whose concentration is even higher for the reverse reaction (whose equilibrium constant is $K_{eq} = 0.24 \pm 0.5$ instead 4.1 for the direct reaction [6–8]).

The calibration curve, necessary for determining the amount of immobilized enzyme from kinetic data, was obtained measuring the initial rate (V_0) employing free fumarase in the concentration range of 0.002–0.37 U/ml (Fig. 3) and a 0.004 M fumarate concentration, corresponding to enzyme saturation with substrate. This makes it possible for a sufficiently high number of measurements to record a high absorbance variation.

Table 1
Values of K_m and V_{max} for the hydration reaction of fumarate and for the dehydration of malate

Substrate		[E] = 0.74 (U/ml)	[E] = 1.48 (U/ml)	[E] = 2.22 (U/ml)	[E] = 2.96 (U/ml)
L-Malate	V_{max} (μM/s)	13 ± 0.5	26.2 ± 0.7	37 ± 1	
	K_m (mM)	2.7 ± 0.6	3.3 ± 0.6	4.3 ± 0.8	
Fumarate	V_{max} (μM/s)	33 ± 2	63 ± 3	91 ± 4	128 ± 8
	K_m (mM)	1.1 ± 0.2	1.5 ± 0.2	1.9 ± 0.3	2.5 ± 0.5

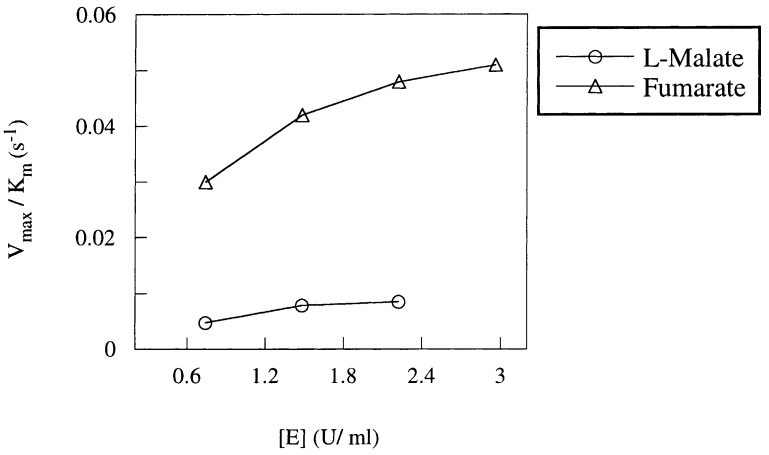

Fig. 2. V_{max}/K_m ratio as a function of the enzyme concentration for both enzymic reactions.

In Table 2 the data relevant to the covalent fumarase immobilization onto EVAL functionalized with AC + EDC are given. Although the immobilization yield is practically quantitative, the performance ratios are very low, and increase slightly with the specific amount of bonded enzyme.

Table 2 also describes the data obtained when fumarase is immobilized on EVAL functionalized by reaction with HMDI and GLA. The performance ratio in this case is better, and it was observed that quantitative immobilization yields can be obtained employing elevated enzyme concentrations (3–4 U/ml). Furthermore, the catalytic activity seems to depend on the steric hindrance: as it is shown in Fig. 4, the former increases with the decrease of the amount of bonded enzyme.

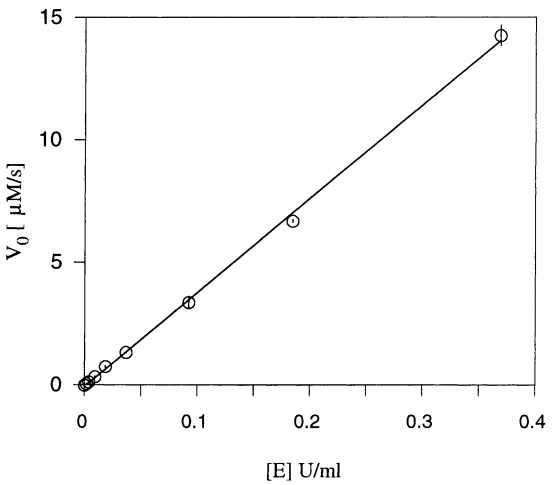

Fig. 3. Calibration curve for determining the amount of immobilized fumarase.

The enzyme activity of the preparations proved also to decrease severely with time: Fig. 5 shows that, after 8 days of operation, the residual activity is only 50%. Also the nature of the polymeric matrix seems to influence the hydration reaction of fumarate: Enzacryl AA, containing aromatic residues and for this reason less hydrophilic than Enzacryl AH, binds a lower amount of enzyme, and also exhibit a lower enzyme activity (Table 2).

The possible inhibition exerted by methanol on the enzyme activity of fumarase covalently bonded to EVAL + HMDI + GLA was investigated using as reaction medium water containing 10 or 20% (v/v) methanol. The enzyme performance ratio, for a methanol content of 10% was, for a preparation employed after 6 days since the immobilization procedure, 47 ± 9% of the initial value, instead of 58 ± 4% (this value was obtained from Fig. 5, where the residual activity versus time is plotted).

Considering the not negligible measurement errors (±4 and ±9%, respectively of standard deviation), it would seem that 10% methanol concentration involves no or very little inhibition. This is confirmed by the fact that, from measurements made after 11 days with the same enzymic preparation, a residual activity of

Table 2
Data relevant to the covalent fumarase immobilization onto functionalized polymers

Sample	Enzyme concentration (U/ml)	Immobilized enzyme (U/g)[a]	Y (%)	Specific activity (U/mg)[b]	η (%)
EVAL + AC + EDC	1.7	25.6	99	0.91	0.61 ± 0.06
EVAL + AC + EDC	15.3	289.7	99	1.49	1.0 ± 0.1
EVAL + HMDI + GLA	3.4	34.3	100	7.30	4.9 ± 0.1
EVAL + HMDI + GLA	0.8	18.3	77	15.32	10.3 ± 0.2
Enzacryl AA	3.6	74.0	75	1.34	0.9 ± 0.3
Enzacryl AH	3.6	72.4	92	3.27	2.2 ± 0.9

[a] U/g: immobilized enzyme units per gram of matrix.
[b] U/mg: active enzyme units per milligram of immobilized protein.

46 ± 8% was obtained, instead of the expected value of 44 ± 3%. This also shows that, if some inhibition takes place, it is completely reversible.

On the contrary, from the activity measurements in a reaction medium containing 20% methanol, after 4 days since the immobilization, a residual activity of 39±7% was observed, instead of the value of 67±3% that could be expected in the absence of inhibition. The same enzyme preparation, tested 6 days after the immobilization in a reaction medium consisting of PBS (without methanol) showed a residual activity of 64 ± 12%, instead of the expected 58 ± 4% value. We conclude that a 20% methanol concentration severely inhibits the enzyme activity, which however, is completely recovered in the absence of the organic solvent. This reversibility had also been observed in a previous work on free fumarase [10].

The fumarase immobilization by physical entrapment into poly(acrylamide) gel gave the most satisfactory results, with quantitative immobilization yields, and irreversible enzyme retention by the polymer network: in fact, no enzyme was detected, after the immobilization reaction, in the washing solutions. Also in this case, the performance ratio decreases with increasing amounts of entrapped enzyme (Fig. 6). An important observation was that, for the highest performance ratio value (about 40%), the enzymic activity does not decrease for at least 15 days (Fig. 7).

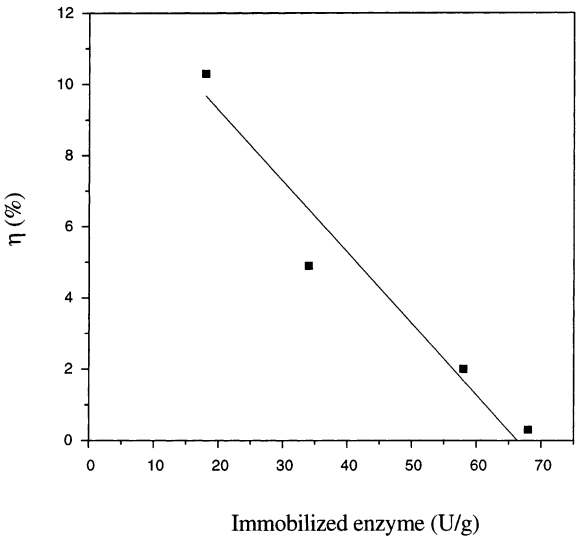

Fig. 4. Performance ratio of fumarase immobilized onto EVAL functionalized with HMDI and GLA, employing different enzyme concentration.

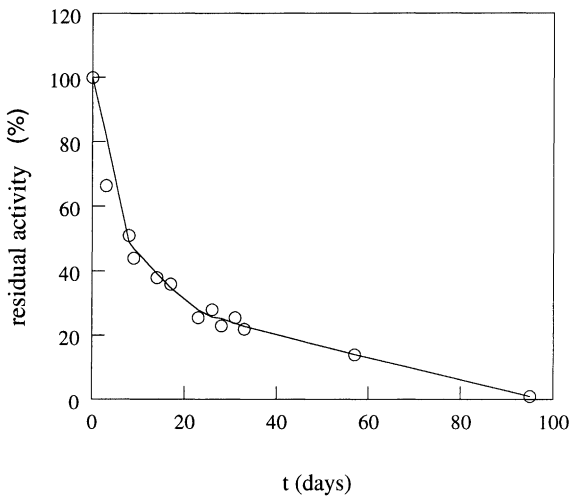

Fig. 5. Enzyme activity of EVAL powders functionalized with HMDI and GLA as a function of the time.

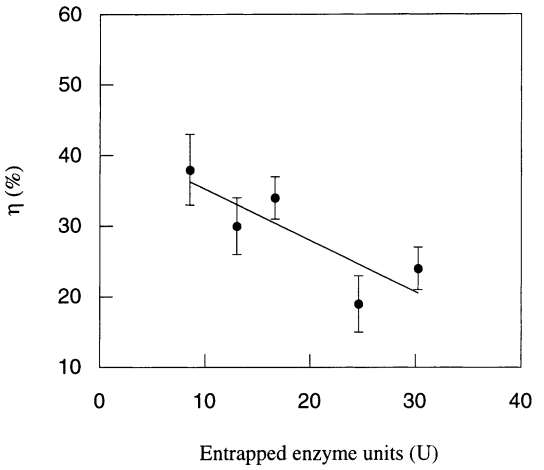

Fig. 6. Performance ratio of immobilized fumarase into poly(acrylamide) gel as a function of entrapped enzyme units.

Also for this type of fumarase immobilization, preliminary tests did not show an influence of methanol on the enzymic activity in the reaction medium, even at a 20% (v/v) concentration.

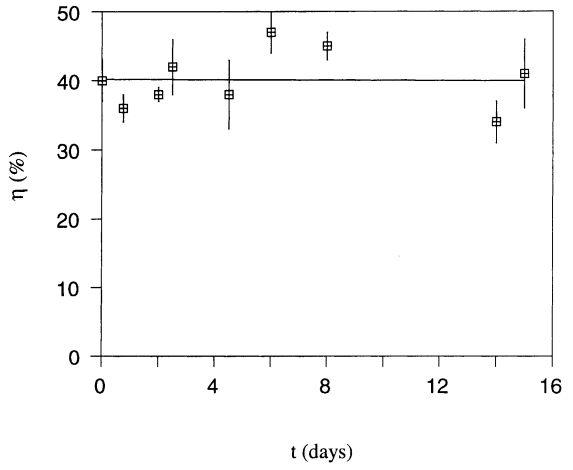

Fig. 7. Enzyme activity of physically entrapped fumarase as a function of the time.

4. Conclusion

The catalytic activity of fumarase, when it is covalently immobilized on polymer surfaces, has proved to be low (few percent units), and to decrease rapidly with time. When less denaturing bifunctional reagents (as glutaraldehyde) are employed, slightly higher performance ratios were obtained, and it was observed their inverse proportionality to the amount of bonded enzyme.

It was also noted that a hydrophilic polymer matrix influences positively both the amount of bonded enzyme and its activity.

It was seen, as it could be expected from the delicate equilibrium involved with the association of the enzyme subunits, due to its quaternary structure, that much higher activity and stability could be obtained by physical entrapment systems, not involving the use of chemical reagents potentially able to modify active site or three-dimensional structure of the enzyme.

By fumarase entrapment in a poly(acrylamide) gel, a quantitative immobilization yield, a performance ratio in the 20–40% range, and a constant activity for at least 15 days were obtained.

It was seen that the hydration reaction of fumarate, catalyzed by covalently immobilized enzyme, is not inhibited by a reaction medium containing 10% methanol (v/v), while a reversible inhibition was observed at a methanol content of 20%. For the physically entrapped enzyme, no inhibition effect was observed even at methanol concentration of 20%.

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