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Influence of the immobilization chemistry on the properties of immobilized β-galactosidases

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Abstract

Neutral β-galactosidases (from *E. coli* and *K. lactis)* were bound to glutaraldehyde-agarose (Glut-agarose) through amino groups, and to thiolsulfinate-agarose (TSI-agarose) through thiol groups. In general, TSI-gels exhibited higher yields after immobilization (60–85%) than Glut-gels (36–40%). The kinetic parameters of the enzymes bound to TSI-gels (particularly those with lower concentration of active groups) were less affected than those of the Glut-gels. This might indicate that the binding to TSI-agarose is more conservative of the protein conformation. However, the Glut-derivatives exhibited in general better thermal and solvent stabilities than TSI-derivatives. The stability of the derivatives was studied in the presence of ethanol, dioxane and acetone (18% v/v). The stabilization of the immobilized enzymes, for some of the solvents assayed, was evidenced by the existence of final very stable enzyme states with high residual activities, thus allowing the utilization of the derivatives in the presence of organic cosolvents.

Keywords: β-Galactosidases; Immobilization; Enzyme stabilization; Solvent stability; Organic solvents

1. Introduction

β-Galactosidases from different sources are currently used in the production of lactose-hydrolyzed milk for lactose intolerant people and in carbohydrate synthetic chemistry [1]. While chemical synthesis of carbohydrates and glycoconjugates requires skilful and selective protection and deprotection reactions, enzymatic processes can provide regio- and stereo-selective products in one step under mild conditions [2]. However, due to their complex molecular structure, biocatalysts are intrinsically unstable. Besides, many applications demand the use of enzymes in reaction mixtures containing organic cosolvents that challenge the stability of the biocatalysts [3]. Therefore, enzyme stability is a key parameter that limits their industrial application [4].

To improve protein stability, several approaches have been developed: the use of stabilizing additives, immobilization, site directed mutagenesis, chemical modification, crosslinking with bifunctional reagents, and the selection of enzymes from thermophilic organisms [4–6]. Immobilization onto solid carriers is perhaps the most used strategy to improve the operational stability of biocatalysts, other benefits being obtained as well, like better operational control, flexibility of reactor design, and ease of product recovery without catalyst contamination [7,8].

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When the effects of enzyme immobilization on the properties of biocatalysts are investigated, a detailed study of the enzyme kinetic properties should be performed. To assess the impact of immobilization on enzyme stability, it is necessary to characterize its deactivation kinetics [5]. The kinetics of enzyme inactivations can be described assuming the existence of partially deactivated states (E_1 and E_2) with nonzero specific activity. The initial state E has a certain specific activity and, if the final state is E_2, we have the following equation

$$E \overset{k_1}{\rightarrow} E_1^{\alpha_1} \overset{k_2}{\rightarrow} E_2^{\alpha_2} \quad (1)$$

Where k_1 and k_2 are first-order deactivation velocity coefficients. A weighed-average activity expression has been derived for a non-zero specific activity for the final state E_2

$$A = \left[100 + \left(\alpha_1 k_1/(k_2 - k_1)\right) - \left(\alpha_2 k_2/(k_2 - k_1)\right)\right]\exp(-k_1 t) - \left(k_1/(k_2 - k_1)\right)(\alpha_1 - \alpha_2) \times \exp(-k_2 t) + \alpha_2 \quad (2)$$

The parameter α_1 is the percentage of the specific activity of the enzyme state E_1 with respect to the enzyme state E $[(E_1/E)*100]$ while α_2 is the corresponding percentage of the specific activity of E_2 with respect to the specific activity of E $[(E_2/E)*100]$ [9].

With $k_2 = 0$ Eq. (1) reduces to Eq. (3)

$$E \overset{k_1}{\rightarrow} E_1^{\alpha_1} \quad (3)$$

Then, the activity-time expression (2) reduces to:

$$A = (100 - \alpha_1)\exp(-k_1 t) + \alpha_1 \quad (4)$$

This is a single-step inactivation which leads to a final state with some residual activity. The enzyme state E_1 is very stable and does not unfold or inactivate to another enzyme state. Since the final state E_1 has some residual activity, this is a possible mechanism to describe enzyme activity stabilization [10]. When the value of α_1 is zero, we have the classical one-step process in which the initially active enzyme state E deactivates to a fully inactive state with zero specific activity, E_d.

β-Galactosidases have been immobilized by several methods to a variety of supports [11,12]. The properties of the derivatives obtained strongly depend on the coupling chemistry and support used. In this work, we have studied the influence of the coupling chemistry on several important characteristics of the biocatalysts obtained: kinetic parameters, stability towards the addition of organic cosolvents and thermal stability. We have selected two coupling chemistries: one based on the reaction of the protein amino groups with aldehyde moieties and the second on the reaction between the protein's thiol groups with disulfide oxide reactive structures on the agarose gel [13,14]. The glutaraldehyde-based chemistry is a very traditional and effective method for immobilization and stabilization [13]. The second method is interesting because even though a covalent bond is formed between enzyme and the matrix, this bond is of disulfide type and can be broken by reaction with reducing agents such as DTT. This fact makes it possible to elute the enzyme when the activity has decayed after use, regenerate the reactive groups on the support, and re-load it with fresh enzyme [14].

2. Materials and methods

β-Galactosidase (β-D-galactoside galactohydrolase; EC 3.2.1.23) grade VI from *E. coli*, epichlorohydrine (1-chloro2,3-epoxy propane), dithiothreitol (DTT), 2,2′- dipyridyl disulfide (2PDS), reduced glutathione, glycidol (2,3 epoxypropanol), sodium periodate, ethylendiamine, 50% glutaraldehyde, *o*-nitrophenyl-β-D-galactopyranoside (ONPG), acetone, ethanol, dioxane and DEAE-Sepharose CL-6B were purchased from Sigma (St. Louis, MO, USA). Maxilact LX 5000, a liquid preparation of yeast lactase derived from *K. lactis*, was kindly supplied by Gist Brocades Food Ingredients (Seclin, Cedex, France). Sepharose 4B was supplied by Pharmacia Biotechnology (Uppsala, Sweden). BCA protein assay reagents were from Pierce (Rockford, IL, USA). Magnesium monoperoxyphtalate was from Fluka (Buchs, Switzerland). All other chemicals used were reagent or analytical grades.

2.1. Matrix activation

2.1.1. Synthesis of thiolsulfinate-agarose

Thiolsulfinate agarose (TSI-gel) containing 2 and 20 μmol TSI/g of suction dried gel (TSI 2 and TSI 20) was prepared as described previously by Batista-Viera et al. [14].

2.1.2. Synthesis of glutaraldehyde-agarose

Glutaraldehyde agarose (Glut-gel) containing 23 and 90 μmol glutaraldehyde/g of suction dried gel (Glut 23 and Glut 90) was prepared as described previously by Guisán et al. [13].

2.2. Protein assay

Protein content of *E. coli* β-galactosidase was determined by using the absorbance value at 280 nm and an extinction coefficient of 2.09 for 1 mg/ml of protein. For the *K. lactis* β-galactosidase, protein was estimated by the bicinchoninic acid (BCA) assay [12]. Immobilized protein was determined as the difference between the amount of protein added to the gel and the protein recovered in the supernatant and washing fractions.

Soluble protein was expressed as mg of protein/ml. Immobilized protein was expressed as mg of protein/g of suction dried gel.

2.3. Enzyme activity

The activity of β-galactosidase was assayed at room temperature and using the chromogen ONPG as substrate. A suitably diluted *K. lactis* enzyme solution was added to 20 mM ONPG in 20 mM potassium phosphate buffer, pH 7.0, containing 0.1 M KCl and 2 mM $MgCl_2$ (*K. lactis* activity buffer). For the *E. coli* β-galactosidase the enzyme solution was assayed using 10 mM ONPG in 50 mM sodium phosphate buffer, pH 7.5, containing 3 mM $MgCl_2$ (*E. coli* activity buffer). The rate of formation of free *o*-nitrophenol (ONP) was recorded spectrophotometrically using a 1-cm path length cuvette provided with magnetic stirring. One unit of enzyme activity (EU) was defined as the amount of enzyme hydrolyzing 1 μmol of substrate per minute in the conditions defined above. Extinction coefficients of 2.0×10^3 M^{-1} cm^{-1} and 3.5×10^3 M^{-1} cm^{-1} for ONP were used for pH 7.0 and pH 7.5, respectively. For the immobilized enzyme, activity was measured under identical conditions by incubating appropriate aliquots of the gel suspensions with the substrate solution mentioned above.

Enzymatic activity was expressed as EU/ml for the soluble enzyme. The gel bound activity was measured directly in the derivative and it was expressed as EU/g of suction dried gel. The specific activity (Sp. Activity) was defined (i) as the ratio between the activity expressed in EU/ml and the concentration in mg of protein/ml, for the soluble enzyme; (ii) as the ratio between the activity expressed in EU/g of suction dried gel and the concentration of immobilized protein in mg/g of suction dried gel, for the immobilized enzyme.

2.4. Reduction of the K. lactis enzyme

The β-galactosidase from *K. lactis* was reduced by the method reported by Ovsejevi et al. [15]. One milliliter of β-galactosidase (Maxilact LX 5000, 24.5 mg/ml, 2426 EU/ml) was incubated for 30 min with 1 ml of 200 mM DTT in 20 mM potassium phosphate, pH 8.0. Excess reducing agent was removed by gel filtration on a Sephadex G-25 column (PD-10).

The thiol content of the enzyme was determined spectrophotometrically, before and after the reduction process, by titration with 2PDS dissolved in 0.1 M sodium phosphate, pH 8.0, by the method of Brocklehurst et al. [16].

2.5. Immobilization of β-galactosidase onto TSI-gel

Aliquots of 1 g suction dried TSI 2 or TSI 20 were incubated with: (i) 10 ml of reduced *K. lactis* β-galactosidase solution (0.41 mg/ml, 43.0 EU/ml) in 0.1 M potassium phosphate buffer, pH 7.0; and (ii) 2.3 ml of *E. coli* β-galactosidase solution (0.89 mg/ml and 62 EU/ml) in 0.1 M sodium phosphate buffer, pH 7.0, containing 3 mM $MgCl_2$. The suspensions were gently agitated at room temperature overnight. Then they were washed in a sintered glass filter with: 0.1 M potassium phosphate, pH 7.0, for the *K. lactis* enzyme; and 0.1 M sodium phosphate,

pH 7.0, containing 3 mM $MgCl_2$, for the *E. coli* enzyme. After this, the β-galactosidase derivative (TSI 2 and TSI 20) were, respectively, incubated with 0.15 mM and 1.5 mM of reduced glutathione in the corresponding activity buffer for 30 min at room temperature. Then they were washed with activity buffer and stored at 4°C.

2.6. Immobilization of β-galactosidase onto glutaraldehyde-agarose

Aliquots of 1 g of suction dried Glut 23 and Glut 90 gels were incubated with: (i) 10 ml of *K. lactis* β-galactosidase solution (0.48 mg/ml, 50.2 EU/ml) in *K. lactis* activity buffer; and (ii) 4 ml of *E. coli* β-galactosidase solution (0.89 mg/ml and 62 EU/ml) in *E. coli* activity buffer. The suspensions were gently agitated at room temperature for 24 h. Then they were washed in a sintered glass filter with the corresponding activity buffer and equilibrated in 40 mM potassium carbonate buffer, pH 10.0, containing 0.1 M KCl and 2 mM $MgCl_2$, for *K. lactis* β-galactosidase; and in 20 mM sodium carbonate, pH 10.0, containing 3 mM $MgCl_2$ for *E. coli* β-galactosidase. Each derivative was suspended in 1 mg/ml sodium borohydride solution in the corresponding carbonate buffer, in a ratio of 1 g of suction dried gel: 14 ml of total volume. The mixtures were gently stirred for 30 min at room temperature. Then they were washed with activity buffer and stored at 4°C.

2.7. Determination of kinetic parameters (K_M and V_{max})

The kinetic parameters were determined using varying concentrations of ONPG: 0.16 to 20 mM for *K. lactis* enzyme and 0.12 to 10 mM for *E. coli* enzyme, in the corresponding activity buffer. The K_M and the V_{max} were determined by the direct linear plot method [17,18].

2.8. Preparation of soluble enzyme extracts for thermal and solvent stabilities

For the *K. lactis* enzyme: 3 g of DEAE-Sepharose CL-6B were equilibrated with 50 mM potassium phosphate buffer, pH 7.0, containing 2 mM $MgCl_2$. An aliquot of 5 ml of *K. lactis* β-galactosidase (243 EU/ml, 2.4 mg/ml) was incubated with the DEAE-Sepharose for 1 h. The DEAE- Sepharose with the adsorbed β-galactosidase was filtered, the supernatant was discarded and the gel was washed with: (i) 20 ml of 50 mM potassium phosphate buffer, pH 7.0, containing 2 mM $MgCl_2$; and (ii) 20 ml of 50 mM potassium phosphate, pH 7.0, containing 2 mM $MgCl_2$ and 50 mM KCl, in fractions of 2 ml. Then the DEAE-Sepharose with the adsorbed β-galactosidase was packed in a column (16 mm diameter) and the protein was eluted with 6 ml of 20 mM potassium phosphate, pH 7.0, containing 2 mM $MgCl_2$ and 0.3 M KCl. The eluate was collected in 2 ml fractions and checked for activity and proteins. The fractions with best specific activity were used for the thermal and solvent stability experiments. The DEAE-Sepharose was regenerated with 50 mM potassium phosphate, pH 7.0, containing 2 mM $MgCl_2$.

The *E. coli* enzyme was gel filtered on a Sephadex G-25 column (PD-10).

2.9. Temperature stability

Aliquots of 2 ml of gel suspensions in the corresponding activity buffer, containing 0.12 mg of protein/ml of suspension, were incubated at 45°C for the *K. lactis* β-galactosidase and 53°C for the *E. coli* β-galactosidase under gentle stirring. Aliquots were taken at regular intervals, brought to room temperature, and the residual activity was determined. Soluble enzyme solutions containing equivalents amounts of protein were treated in the same way.

2.10. Stability towards organic solvents

Aliquots of 1.64 ml of gel suspensions in the corresponding activity buffer, containing 13 EU/ml for the *K. lactis* derivatives and 9 EU/ml for the *E. coli* derivatives, were incubated with 0.36 ml of the organic solvents at 30°C (final cosolvent concentration: 18% v/v). At intervals, samples were taken for activity determination and the residual activity was plotted against time of exposure. Soluble enzyme

solutions containing equivalent amount of EU/ml were treated in the same way. The solvents used were: ethanol, acetone and dioxane.

The experiments were performed in triplicate.

3. Results

3.1. Immobilization of β-galactosidases

The coupling chemistries used for the immobilization of β-galactosidases to agarose gels were based on the reaction with glutaraldehyde and thiolsulfinate gel bound groups. The results of the protein and activity immobilization yields are shown in Table 1.

In the case of *K. lactis* β-galactosidase-derivatives, increasing the number of reactive glutaraldehyde groups from 23 to 90 μmol/g of suction dried gel allowed to achieve a better protein immobilization yield (from 63% to 83%, Table 1a). For both, Glut 23 and Glut 90 nonreduced derivatives, immobilization was attained expressing approximately 60% of the applied activity. However, the reducing treatment necessary to block excess reactive groups on the gel led to a 30%–40% decrease in gel bound activity. Changing the reaction time from 24 to 5 h did not affect the protein or activity yields upon immobilization.

The degree of activation of the matrix with TSI groups was a very important parameter for the immobilization of *K. lactis* β-galactosidase. In this case, by increasing the number of reactive groups from 2 to 20 μmol/g of suction dried gel, the protein immobilization yield improved from 16% to 61% with a concomitant gain in the activity yield from 12% to 60%.

The *E. coli* Glut 90-derivative was very similar to the corresponding *K. lactis* Glut 90 with respect to protein and activity immobilization yields (Table 1). The gel-bound activity of the *E. coli* β-galactosidase bound to TSI-agarose was higher than the corresponding values for the *K. lactis* enzyme. For the *E. coli* TSI derivatives increasing the number of reactive groups from 2 to 20 μmol/g of suction dried only attained a minor increment in activity immobilization yield from 75% to 85%. Interestingly, the TSI gels displayed high selectivity towards the *E. coli* enzyme and, therefore, the immobilization proceeded with a concomitant purification. The specific

Table 1
Immobilization of β-galactosidase (*E. coli* and *K. lactis*) on thiolsulfinate-agarose and glutaraldehyde-agarose

Gel	Reaction time (h)	Reduction treatment	Gel-bound protein		Gel-bound activity		Gel-bound Sp. Activity (EU/mg)	K_M (mM)	V_{max} (μmol ONP/min mg)
			mg/g gel	(%)[a]	EU/g gel	(%)[b]			
(a) K. lactis-β-galactosidase derivatives									
Glut 23	24	No	2.8	63	298	64	106	7.6	62
Glut 23	24	Yes	2.8	63	178	38	64	5.2	49
Glut 90	24	No	4.3	83	281	57	65	4.6	40
Glut 90	24	Yes	4.3	83	197	40	46	3.5	37
Glut 90	5	Yes	3.7	81	177	36	48	3.5	32
TSI 2	24	–	0.6	16	51	12	85	2.3	72
TSI 20	24	–	2.0	61	217	60	109	4.9	88
(b) E. coli β-galactosidase derivatives									
Glut 90	24	Yes	2.3	78	91	39	40	2.2	70
TSI 2	24	–	0.7	29	75	75	94	0.4	64
TSI 20	24	–	0.8	46	130	85	150	0.8	105

Soluble enzyme (*K. lactis*): specific activity: 99 EU/mg, K_M: 2.0 mM, V_{max}: 91 μmol ONP/min mg.
Soluble enzyme (*E. coli*): specific activity: 70 EU/mg, K_M: 0.13 mM, V_{max}: 84 μmol ONP/min mg.
The results are expressed in mg of protein or EU per gram of suction dried gel.
[a]Amount of immobilized protein as percentage of the amount of applied protein (protein immobilization yield).
[b]Amount of immobilized activity as percentage of the amount of applied activity (activity immobilization yield).

activity of the immobilized enzyme was increased by factors of 1.3 and 2.1 for TSI 2 and TSI 20, respectively.

In general, TSI-gels exhibited a higher percentage of gel-bound activity (60%–85%) than glutaraldehyde-gels (40%). Moreover, the immobilization of β-galactosidases onto glutaraldehyde-agarose resulted in an inactivation of the enzyme, which is reflected in the decrease of the specific activity of the derivatives (1.5- to 2.1-fold for the reduced derivatives).

The kinetic parameters of the enzymes bound to TSI-gels and particularly to the gels with lower concentration of active groups were less affected than those bound to glutaraldehyde-gels, compared to the soluble enzymes.

3.2. Thermal stability

The results of the thermal inactivation parameters of the β-galactosidase biocatalysts are shown in Table 2. The thermal deactivation curves of the soluble and immobilized enzyme derivatives were analyzed according to the two-step deactivation model proposed by Henley and Sadana [9]. The experimental plots of residual activity versus time at a fixed temperature were adjusted to exponential decays, simple or double, with or without offset (Eqs. 2 and 4) with the help of the Enzfitter program. From the data adjusted to Eq. (2), we calculated the parameters: k_1, k_2, α_1, α_2 and the half-life of the biocatalysts.

The half-life of the soluble *K. lactis* enzyme was 0.4 h at 45°C and as shown by the increased half life times and the higher value of the parameter α_1 (Table 2), all the immobilized derivatives showed some degree of stabilization. The thermal deactivation parameters of both glutaraldehyde derivatives (24 h reaction time, reduced) were very similar to each other, both of them presented α_1 values close to 50% with k_2 values of zero. The reduction process was necessary to increase the value of α_1 (from 21% to 50–53%). The reaction time also plays an important role in enzyme stability; a change from 24 to 5 h decreases the value of α_1 from 53% to 27%. Therefore, we selected the Glut 90-derivative synthesized by 24 h of incubation time and subsequent reduction to continue with the characterization of the derivatives. Even though the half-life of the TSI 20-derivative is lower than that of the TSI 2-derivative, the former has a higher α_1 and, thus, is more stable from that point of view. We, therefore, selected the TSI 20-derivative for further studies on the basis of its higher activity and thermal stability.

In the case of the *E. coli* derivatives, immobilization also had a positive effect on thermal stability (Table 2). The Glut 90-derivative presented the best

Table 2
Thermal deactivation parameters from *K. lactis* and *E. coli* β-galactosidase derivatives at 45°C and 53°C, respectively

	Reation time (h)	Reduction treatment	k_1 (h^{-1})	k_2 (h^{-1})	α_1	α_2	t 1/2 (h)
K. lactis derivatives							
Soluble enzyme	–	–	2.4	0	13	0	0.4
Glut 23	24	No	1.1	0	21	0	0.9
Glut 23	24	Yes	1.9	0	50	0	–
Glut 90	24	No	1.3	0	21	0	0.8
Glut 90	24	Yes	2.5	0	53	0	–
Glut 90	5	Yes	3.4	0	27	0	0.5
TSI 2	24	–	0.5	0	15	0	2.8
TSI 20	24	–	1.1	0	30	0	1.2
E. coli derivatives							
Soluble enzyme	–	–	20.7	0.60	58	0	0.3
Glut 90	24	Yes	15.2	0.48	77	0	0.9
TSI 20	24	–	18.1	0.53	66	0	0.4

Table 3
Deactivation parameters from *K. lactis* β-galactosidase derivatives in cosolvents systems

Cosolvent	Derivative	k_1 (h^{-1})	k_2 (h^{-1})	α_1	α_2	t 1/2 (h)
Buffer	Soluble	7.6×10^{-3}	0	0	0	91
	TSI 20	8.1×10^{-3}	0	0	0	86
	Glut 90	5.1×10^{-3}	0	0	0	137
Ethanol 18%	Soluble	8.1×10^{-3}	0	0	0	87
	TSI 20	7.2×10^{-3}	0	0	0	97
	Glut 90	4.1×10^{-2}	0	49	0	86
Dioxane 18%	Soluble	1.0×10^{-1}	0	0	0	7
	TSI 20	1.3×10^{-1}	0	0	0	5
	Glut 90	1.2	5.8×10^{-2}	76	0	8
Acetone 18%	Soluble	2.6×10^{-2}	0	0	0	26
	TSI 20	2.7×10^{-1}	0	43	0	8
	Glut 90	1.4×10^{-1}	0	46	0	19

thermal stability both from the point of view of the half-life time and the deactivation constants k_1 and k_2.

3.3. Deactivation in the presence of organic cosolvents

Tables 3 and 4 show the parameters that characterize the deactivation of the different *K. lactis* and *E. coli* biocatalysts in the presence of organic cosolvents.

In buffer at 30°C, the deactivation kinetics of both the immobilized and soluble enzyme followed a single exponential decay which corresponds to the classical first order deactivation process with $\alpha_1 = 0$, $\alpha_2 = 0$, and $k_2 = 0$. The stabilizing effect of immobilization at 30°C was stronger for the *E. coli* enzyme as evidenced by the increase in the half-life of both derivatives in buffer (8.5-fold for Glut 90 and 4-fold for TSI 20) and by the decrease in the deactivation constant k_1 (Tables 3 and 4). In general, all the cosolvents tested had a deleterious effect on the stability of the soluble and immobilized derivatives compared to their performance in buffer.

The deactivation kinetics of *K. lactis* biocatalysts in the presence of ethanol 18% (v/v) are similar to those observed in buffer (Table 3). An exception to

Table 4
Deactivation parameters from *E. coli* β-galactosidase derivatives in cosolvent systems

Cosolvent	Derivative	k_1 (h^{-1})	k_2 (h^{-1})	α_1	α_2	t 1/2 (h)
> Buffer	Soluble	1.2×10^{-3}	0	0	0	590
	TSI 20	3.0×10^{-4}	0	0	0	2300
	Glut 90	1.4×10^{-4}	0	0	0	5000
Ethanol 18%	Soluble	5.2	8.2×10^{-3}	87	0	55
	TSI 20	3.6×10^{-2}	0	54	0	–
	Glut 90	3.2×10^{-2}	0	73	0	–
> Dioxane 18%	Soluble	1.4×10^{-1}	0	27	0	8
	TSI 20	2.4×10^{-1}	0	0	0	3
	Glut 90	7.1×10^{-3}	0	0	0	100
Acetone 18%	Soluble	6.7×10^{-2}	0	76	0	–
	TSI 20	1.9×10^{-1}	0	42	0	11
	Glut 90	8.0×10^{-3}	0	22	0	130

this was the Glut 90-derivative which changed from a first-order deactivation kinetics to a biphasic mechanism with a stable state E_1 exhibiting a residual activity $\alpha_1 = 49\%$. The high value of α_1 evidences the stabilization achieved by the Glut 90-derivative.

Dioxane 18% (v/v) had a strong detrimental effect on the stability of *K. lactis* β-galactosidase and, in this case, it was not possible to observe any stabilizing effect of immobilization.

The incubation of the soluble β-galactosidase with acetone 18% (v/v) presented a single exponential decay with a half-life time of 26 h, which is significantly lower than the corresponding value in buffer. The inactivation kinetics of both immobilized derivatives changed to a mechanism of single exponential decay with offset (Table 3, Fig. 1). The stabilization of the Glut 90 and TSI 20 derivatives is, therefore, shown by the value of α_1 (46% and 43%, respectively).

Upon exposure of the soluble *E. coli* β-galactosidase to 18% (v/v) ethanol, the enzyme changed from first-order inactivation decay shown in buffer to a biphasic mechanism (Table 4, Fig. 2). However, the highly active intermediate state E_1 rapidly decays to the fully deactivated E_2 state and, thus, the half-life of the enzyme is decreased by a factor of 11 with respect to the corresponding value in buffer. The immobilization had a strong stabilizing effect

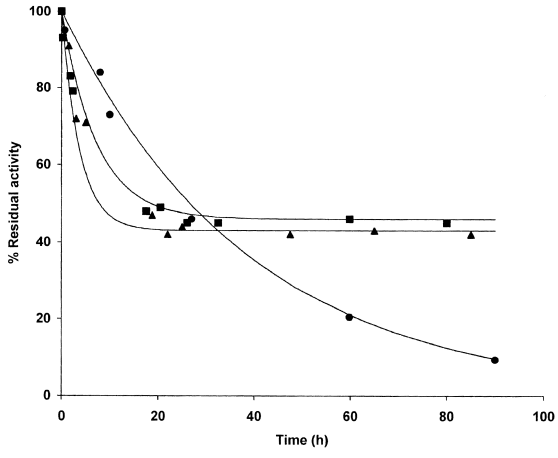

Fig. 1. Stability towards acetone 18% (v/v) for *K. lactis* β-galactosidase: ● Soluble enzyme; ▲ TSI 20-derivative; ■ Glut 90-derivative.

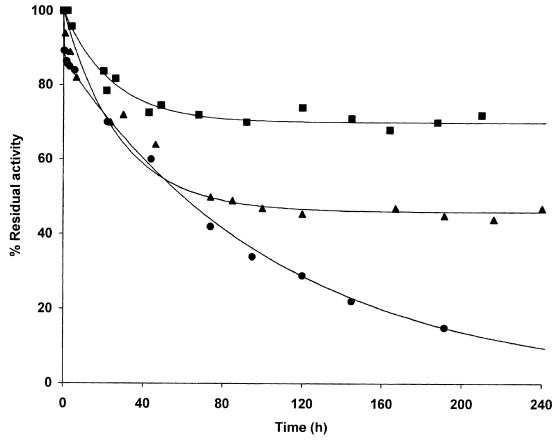

Fig. 2. Stability towards ethanol 18% (v/v) for *E. coli* β-galactosidase: ● Soluble enzyme; ▲ TSI 20-derivative; ■ Glut 90-derivative.

for both the Glut 90 and TSI 20 derivatives as shown by the existence of the very stable intermediate state E_1 with high residual activity (73% for Glut 90 and 54% for TSI 20) (Fig. 2).

The incubation of soluble enzyme with dioxane 18% (v/v) promoted a change of its deactivation kinetics with respect to the behavior in buffer; a stable state E_1 with a residual activity α_1 was established ($\alpha_1 = 27\%$). Even though both enzyme derivatives presented an α_1 value of 0, immobilization on Glut 90 improved the stability of the enzyme owing to the lower value of k_1 (20-fold lower than for the soluble enzyme). The stabilization of the Glut 90-derivative was also shown by the increase of the half-life from 8 to 100 h.

The analysis of the data in acetone 18% (v/v) shows a change in the deactivation kinetics from a single exponential decay in buffer to a mechanism with a stable state E_1. In this case the, soluble enzyme was very stable due to its high value of α_1 (76%); the activity of the E_1 states was lower for both immobilized derivatives.

4. Discussion

The effect of the coupling method on the properties of the different β-galactosidase biocatalysts has

been evaluated through the determination of protein and activity yields, the kinetic parameters K_M and V_{max}, as well as the thermal stability. In order to study the possible application of the derivatives for synthetic chemistry, the inactivation parameters in the presence of several water-soluble organic solvents was characterized. Enzyme stabilization has been defined in different terms. The determination of half-life time under certain conditions is often chosen, since it provides useful information about residual activity regarding their applications. In our case, we refer to enhanced enzyme stability as a decrease in the deactivation parameters k_1 and k_2 (and a corresponding increase in half life) or as an increase in enzyme residual activity α_1.

The coupling chemistry has been shown to be an important parameter determining the properties of immobilized β-galactosidases from *K. lactis* and *E. coli* (Table 1). The higher activity immobilization yield obtained for the TSI derivatives is most likely the result of the mild conditions used throughout this immobilization procedure. For the case of the Glut-derivatives, the high pH values during the reducing treatment negatively affected the gel-bound activity and the V_{max} values. However, this decrease is offset by a gain in thermal stability (Table 2), therefore, the reducing treatment should not be avoided.

Immobilization often leads to an important increase in K_M values with respect to those of the soluble enzymes. In the case of the *K. lactis* enzyme, the higher K_M values are observed for the higher gel-bound activity, probably indicating the contribution of diffusional limitations on substrate and products. The Glut 90 *E. coli* derivative has a high K_M value together with a low V_{max} which could be due to a conformational change among other factors. The kinetic parameters of TSI derivatives are less affected than those of the Glut derivatives for both enzymes studied. This may indicate that protein conformation is better preserved when bound to the TSI than to the Glut gels. However, in general the thermal stability of the Glut-derivatives is better than the stability of the corresponding TSI-derivatives, so this is again a situation in which some activity is lost in favor of an increased stability. The better stability of the Glut derivatives may be due to the fact that glutaraldehyde is known to polymerize and, thus, is prone to introduce a number of cross links between the abundant lysyl groups on the enzymes and the aldehyde moieties on the matrix [19]. The introduction of cross links leads to a rigidification of the tertiary structure and this is recognized as a general mechanism for stabilization of the native conformation [9]. Native soluble *E. coli* β-galactosidase withstands higher temperatures than the enzyme from *K. lactis* but it was more difficult to stabilize by immobilization by both the procedures used. The fact that the enzyme from *E. coli* is tetrameric and the one from *K. lactis* is dimeric may account for this situation because it is very unlikely that all the subunits of a tetrameric enzyme will bind to the matrix.

The use of enzymes in reaction mixtures containing organic cosolvents for industrial processes is a very active field of research. Organic cosolvents are used to shift equilibrium towards the synthetic process instead of hydrolysis and to dissolve substrates. Nonpolar organic solvents are poor solvents for polar substrates such as carbohydrates. On the other hand, polar organic solvents are usually very aggressive and enzymatic inactivation increases with the polarity of the solvent. Therefore, the stability of biocatalysts is a key parameter that limits their industrial application, and enzyme stabilization is a central issue of biotechnology [4].

Upon incubation with organic cosolvents the native soluble *K. lactis* enzyme exhibits single exponential decay with no residual activity and this behavior changed for most of the immobilized derivatives (Table 3). In 18% (v/v) ethanol, stabilization was evidenced by the existence of a high residual activity α_1 for the Glut 90-derivative and a decreased value of k_1 for the TSI 20-derivative. Although the half-life time is longer for the TSI 20-derivative, the Glut 90-derivative should be preferred for use in the presence of 18% (v/v) ethanol due to the high activity and stability of the enzyme state E_1 ($\alpha_1 = 49\%$, $k_2 = 0$). Use of 18% (v/v) acetone should also be acceptable with both derivatives since again the intermediate state E_1 is highly active and stable (Table 3, Fig. 1).

The native soluble *E. coli* enzyme is in general more stable to solvents than the one from *K. lactis*, however, it proved to be more difficult to stabilize (Table 4, Fig. 2). Also for the *E. coli* biocatalysts, Glut 90-derivative is more stable than the TSI 20-de-

rivative and due to this it should be feasible to use them in the presence of the cosolvents studied.

It is interesting to note that of the three solvents studied, dioxane was the most aggressive and it was the only one in the presence of which there was no stable residual enzyme activity for the immobilized biocatalysts. Nevertheless, the half-life of the *E coli* Glut derivative was increased 12-fold when compared to the soluble enzyme.

5. Conclusions

The first-order deactivation kinetics may be attributed to the occurrence of a single lethal event or "single hit" and it implies a highly cooperative transition between the active and the denatured state. The change from this behavior to more complex kinetics involving a higher number of parameters may be interpreted as an indication of enzyme stabilization. Most of our experimental data fitted well to a two-phase series mechanism Finally, though TSI derivatives had a higher yield of immobilization, Glut derivatives exhibited better thermal and solvent stability properties, which make them more suitable for biocatalysis under aggressive conditions. This highlights the relevance of the immobilization chemistry for application-oriented design of biocatalysts.

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References

[1] J. Thiem, FEMS Microbiol. Rev. 16 (1995) 193.
[2] E. Polastro, Bio/Technology 7 (1989) 1238–1241.
[3] G. Irazoqui, A. Villarino, F. Batista-Viera, B.M. Brena, Biotechnol. Tech. 12 (1998) 885–888.
[4] A. Illanes, http://www.ejb.org/content/vol2/issue1/full/2/.
[5] M.A. Longo, D. Combes, in: A. Ballesteros, F.J. Plou, J.L. Iborra, P.J. Halling (Eds.), Stability and Stabilization of Biocatalysts, Elsevier, Amsterdam, 1998, 135.
[6] H. Noritomi, K. Koyama, S. Kato, K. Nagahama, Biotechnol. Tech. 12 (1998) 467–469.
[7] E. Katchalsky-Katzir, Trends Biotechnol. 11 (1993) 471–478.
[8] J.M. Guisan, R. Fernandez-Lafuente, V. Rodriguez, A. Bastida, G. Alvaro, in: W. Van den Tweel, A. Harder, R. Buitelaar (Eds.), Stability and Stabilization of Enzymes, Elsevier, Amsterdam, 1993, pp. 55–62.
[9] J.P. Henley, A. Sadana, Biotechnol. Bioeng. 26 (1984) 959–969.
[10] A. Sadana, J.P. Henley, Biotechnol. Bioeng. 30 (1987) 717–723.
[11] T.P. Shukla, CRC Crit. Rev. Food Sci. Nutr. 5 (1975) 325–356.
[12] C. Giacomini, A. Villarino, L. Franco-Fraguas, F. Batista-Viera, J. Molec. Catal. B: Enzym. 4 (1998) 313–327.
[13] J.M. Guisán, G. Penzol, P. Armisen, A. Bastida, R.M. Blanco, R. Fernandez-Lafuente, E. García-Junceda, in: G.F. Bickerstaff (Ed.), Immobilization of Enzymes and Cells, Humana Press, Clifton, NJ, USA, 1997, p. 261.
[14] F. Batista-Viera, C. Manta, J. Carlsson, Appl. Biochem. Biotechnol. 44 (1994) 1–14.
[15] K. Ovsejevi, V. Grazú, F. Batista Viera, Biotechnol. Techn. 12 (2) (1998) 143–148.
[16] K. Brocklehurst, J. Carlsson, M. Kierstan, E. Crook, Biochem. J. 133 (1973) 573–584.
[17] J. Peter, J.E. Prenosil, J.R. Bourne, J. Chem. Tech. 8 (4) (1994) 233.
[18] A.T. Cornish-Bowden, in: A.T. Cornish-Bowden (Ed.), Fundamentals of Enzyme Kinetics, Portland Press, London, 1995, p. 297.
[19] R.A. Messing, in: R.A. Messing (Ed.), Immobilized Enzymes for Industrial Reactors, Academic Press, New York, 1975, p. 104.