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Hydrophilization of immobilized model enzymes suggests a widely applicable method for enhancing protein stability in polar organic co-solvents

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Abstract

β-Galactosidases from *Escherichia coli*, *Kluyveromyces lactis* and *Aspergillus oryzae* were used to characterize the potential for enzyme stabilization of a two-step strategy: (i) immobilization on glutaraldehyde-agarose (Glut90), (ii) subsequent generation of a hydrophilic nano-environment by reaction with polyaldehyde-dextran polymer (Glut90-Pal), followed by polyamine-dextran polymer (Glut90-Pal-Pam). The derivatives were characterized by kinetics parameters, co-solvent (ethanol and acetone) and temperature stability. Hydrophilization achieved important co-solvent stabilization in all cases. One of the most remarkable results obtained was a 25-fold increase in the half-life of the *A. oryzae* Glut90-Pal-Pam derivative in 50% (v/v) acetone. Stabilization achieved in very drastic co-solvent concentrations is directly related to the hydrophilization of the nano-environment. The K_M values show that the hydrophilic shell appears to behave as an open structure and may create a "partition effect" that protects the enzymes from denaturation. These results show the potential of hydrophilization for building up additional stabilization of immobilized enzymes which would make possible the development of industrial applications.

Keywords: β-Galactosidase; Enzyme stabilization; Nano-environment; Solvent stability; Hydrophilization; Organic co-solvents

1. Introduction

The use of organic solvents and co-solvents widens the possible applications of many enzymes that are useful for carrying out biotransformations [1]. A well-known example is that of the glycosidases which, in the presence of an acceptor molecule (alcohol or saccharide) are capable of trapping the glycosyl intermediate allowing the synthesis of glycosides or oligosaccharides. In order to achieve the formation of glycosidic linkage, manipulations of the reaction system are necessary, e.g., by adding organic co-solvents and lowering the water activity [2,3]. Thus, the use of water/co-solvent media allows hydrophobic compounds to enter into solution, which can shift thermodynamic equilibria towards synthesis or other desired outcomes [4]. However, enzyme molecules are usually very unstable under such experimental conditions. This lack of stability severely limits the industrial implementation of such interesting biotransformation reactions in non-conventional media [5,6].

Protein molecules in solution are surrounded by a hydration shell composed of water molecules attached to the protein surface mainly by hydrogen bonds. This hydration shell is indispensable for maintaining the native protein conformation. If a polar organic solvent is present in solution, its molecules tend to displace water from the hydration shell, distorting the interactions responsible for keeping the enzyme molecule in its native conformation, and thus, may finally unfold the protein [7,8]. It is generally accepted that the destruction of the hydration shell is one of the main causes of protein denaturation by organic solvents [9,10].

A rational approach to the protection of proteins from such denaturation is therefore to promote a drastic reduction of the co-solvent concentration in the immediate vicinity of the enzyme molecules. In this way, there are several strategies reported such as: cross-linked enzyme crystals (CLEC) [11,12], cross-linked enzyme aggregates (CLEA) [13,14], reverse micelles [15,16], entrapment of the protein molecule in a strongly hydrophilic

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matrix [17]. Other methods to improve stability in the presence of organic solvents include molecular methods such as site-directed mutagenesis or directed evolution [5,18], chemical modifications with hydrophilic compounds [19,20], and immobilization onto solid-phase supports [21]. We have previously reported improvement of stability of β-galactosidases at low concentrations of organic co-solvents [22] by means of protein immobilization on to glutaraldehyde-agarose. However, when the co-solvent concentrations in the mixture were increased, the immobilized derivatives were in some cases less stable than the enzymes in solution [23].

The combination of immobilization of the protein and hydrophilization of its nano-environment has proved a useful strategy for achieving stabilization in the presence of co-solvents of a few enzymes such as penicillin G acylase [24], lipases [25] and pig liver esterase [26]. Therefore, we decided to characterize this two-step strategy using a series of three different β-galactosidases as a model system, in order to evaluate its possible application as a general stabilization method for enzymes under drastic organic co-solvent concentration conditions. Since β-galactosidase-mediated transglycosylation procedures in the presence of water-miscible organic solvents have been applied to the synthesis of β-D-galactosyl disaccharides and *n*-alkyl β-D-galactopyranosides [2,27,28], an improvement in stability properties would allow the development of industrial applications.

2. Materials and methods

All results represent averages of at least three experiments.

2.1. Materials

β-Galactosidase (β-D-galactoside galactohydrolase; EC 3.2.1.23) from *Escherichia coli* (grade VI) and from *Aspergillus oryzae*, glycidol (2,3-epoxypropanol), sodium periodate, ethylenediamine, 50% glutaraldehyde, *o*-nitro-phenyl-β-D-galactopyranoside (ONPG), dextran of average MW 41 kDa and 71 kDa, acetone, ethanol and trimethylamineborane (TMAB) were purchased from Sigma (St. Louis, MO, USA). Maxilact LX 5000, a liquid preparation of yeast lactase derived from *Kluyveromyces 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). All other chemicals used were reagent or analytical grade.

2.2. Synthesis of glutaraldehyde-agarose

Glutaraldehyde-agarose containing 90 μmol glutaraldehyde/g of suction-dried gel were prepared as described previously by Guisán et al. [29].

2.3. Protein assay

Protein content of the soluble and immobilized enzyme was estimated by the bicinchoninic acid (BCA) assay [30]. Immobilized protein was expressed as milligrams of protein per gram of suction-dried gel.

2.4. Enzyme activity

The activity of β-galactosidase was assayed at room temperature using the chromogen ONPG as substrate. A suitably diluted *E. coli* β-galactosidase 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). For the *K. lactis* enzyme a suitably diluted 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 *A. oryzae* β-galactosidase a suitably diluted enzyme solution was added to 25 mM ONPG in 50 mM sodium acetate buffer, pH 5.5 (*A. oryzae* activity buffer). The rate of formation of free *o*-nitrophenol (ONP) was recorded spectrophotometrically at 405 nm 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 substrate min^{-1} in the conditions defined above. Extinction coefficients of $7.5 \times 10^2\ M^{-1}\ cm^{-1}$, $2.0 \times 10^3\ M^{-1}\ cm^{-1}$ and $3.5 \times 10^3\ M^{-1}\ cm^{-1}$ for ONP were used for pH 5.5, 7.0 and 7.5, respectively. For the immobilized enzymes, activity was measured under identical conditions by incubating appropriate aliquots of the gel suspensions with the substrate solutions and activity buffers mentioned above.

Enzymatic activity was expressed as EU per milliliter for the soluble enzyme and as EU per gram of suction-dried gel for the gel-bound activity of the derivative.

2.5. Immobilization of β-galactosidase on to glutaraldehyde-agarose

Aliquots of 1 g of suction-dried glutaraldehyde-agarose containing 90 μmol glutaraldehyde/g of suction-dried gel were incubated with: (i) 4 ml of *E. coli* β-galactosidase solution (1.6 mg/ml and 72 EU/ml) in *E. coli* activity buffer; (ii) 10 ml of *K. lactis* β-galactosidase solution (1.3 mg/ml and 12 EU/ml) in *K. lactis* activity buffer; and (iii) 10 ml of *A. oryzae* β-galactosidase solution (2 mg/ml, 94 EU/ml) in 50 mM sodium phosphate pH 7.0. The suspensions were gently agitated at room temperature for 24 h. Then they were washed in a sintered glass filter with the appropriate activity buffer and equilibrated in (i) 20 mM sodium carbonate, pH 10.0, containing 3 mM $MgCl_2$ for *E. coli* β-galactosidase, (ii) 40 mM potassium carbonate buffer, pH 10.0, containing 0.1 M KCl and 2 mM $MgCl_2$ for *K. lactis* β-galactosidase, and (iii) 20 mM sodium carbonate, pH 10.0 for *A. oryzae* β-galactosidase. Each derivative was suspended in 26.4 mM sodium borohydride solution in the appropriate carbonate buffer, at 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 the appropriate activity buffer and stored at 4 °C. These enzyme derivatives were named Glut90 derivatives.

2.6. Preparation of polyaldehyde dextran

Aldehyde dextrans (MW 41 kDa and 71 kDa) were obtained by total oxidation of dextrans with sodium periodate as previously reported by Guisán et al. [31].

2.7. Preparation of polyamine dextran

Amine-dextran (MW 71 kDa) was prepared by reaction of aldehyde dextran with ethylenediamine and further reduction with sodium borohydride as previously described [31].

2.8. Modification of the immobilized enzyme with polyaldehyde dextrans

Aliquots of 1 g of suction-dried Glut90 gel derivative were suspended in 4 ml of: (i) 15 mM sodium phosphate buffer, pH 7.0, containing 3 mM $MgCl_2$ and 200 mM TMAB for the *E. coli* enzyme derivative; (ii) 15 mM potassium phosphate buffer, pH 7.0, containing 0.1 M KCl, 2 mM $MgCl_2$ and 200 mM TMAB for the *K. lactis* enzyme derivative; (iii) 15 mM sodium phosphate buffer, pH 7.0 containing 200 mM TMAB for the *A. oryzae* enzyme derivative. Suitable aliquots of polyaldehyde-dextran solution were added to each gel suspension so that the molar ratio of polyaldehyde dextran to enzyme was 10:1. The reaction mixtures were incubated at room temperature under very gentle stirring for 24 h. At that time the gel derivatives were filtered and washed with activity buffer. Then, Schiff's bases were reduced to secondary amine bonds, between the amine groups from the enzyme surface and the aldehyde groups from the dextran polymer. To perform this reduction, the gel aliquots were suspended in 9.6 ml of: (i) 40 mM sodium carbonate buffer pH 10 containing 3 mM $MgCl_2$ for the *E. coli* enzyme derivative; (ii) 40 mM potassium carbonate buffer pH 10 containing 0.1 M KCl, 2 mM $MgCl_2$ for the *K. lactis* enzyme derivative; (iii) 40 mM sodium carbonate buffer pH 10 for the *A. oryzae* enzyme derivative. The suspensions were supplemented with sodium borohydride to a final concentration of 26.4 mM, and were incubated at room temperature with gentle stirring for 30 min. After that the gel derivatives were filtered and washed exhaustively with the appropriate activity buffer and stored at 4 °C. These enzyme derivatives were named Glut90-Pal.

2.9. Modification of the immobilized enzyme with polyamine dextran

Aliquots of 1 g of suction-dried Glut90-Pal gel derivative which had not been subjected to the final reduction step with sodium borohydride but otherwise made as above, were suspended in 4 ml of: (i) 15 mM potassium phosphate buffer, pH 7.0, containing 0.1 M KCl, 2 mM $MgCl_2$ and 200 mM TMAB for the *K. lactis* enzyme derivative, or (ii) 15 mM sodium phosphate buffer, pH 7.0 containing 200 mM TMAB for the *A. oryzae* enzyme derivative. Suitable aliquots of polyamine dextran solution were added to each gel suspension, so that the molar ratio of polyamine dextran to enzyme was 1:1. The reaction mixtures were incubated at room temperature under very gentle stirring for 24 h. At that time the gel derivatives were filtered and washed with the appropriate activity buffer. Then reduction was carried out: the gel aliquots were suspended in 9.6 ml of: (i) 40 mM potassium carbonate buffer pH 10.0 containing 0.1 M KCl, 2 mM $MgCl_2$ for the *K. lactis* enzyme derivative; or (ii) 40 mM sodium carbonate buffer pH 10.0 for the *A. oryzae* enzyme derivative. The suspensions were supplemented with sodium borohydride to a final concentration of 26.4 mM, and were incubated at room temperature with gentle stirring for 30 min. After that the gel derivatives were filtered and washed exhaustively with the appropriate activity buffer and stored at 4 °C. These enzyme derivatives were named Glut90-Pal-Pam.

2.10. SDS-PAGE analysis of the immobilized enzymes' quaternary structure

Aliquots of enzyme-gel derivatives containing a protein concentration of 1 mg/ml were boiled in the presence of mercaptoethanol and SDS. The supernatants were analyzed by SDS-PAGE on 12% polyacrylamide gels using Phast System equipment (Pharmacia, Uppsala). Proteins were silver stained according to the manufacturer's instructions.

2.11. Stability in the presence of organic solvents

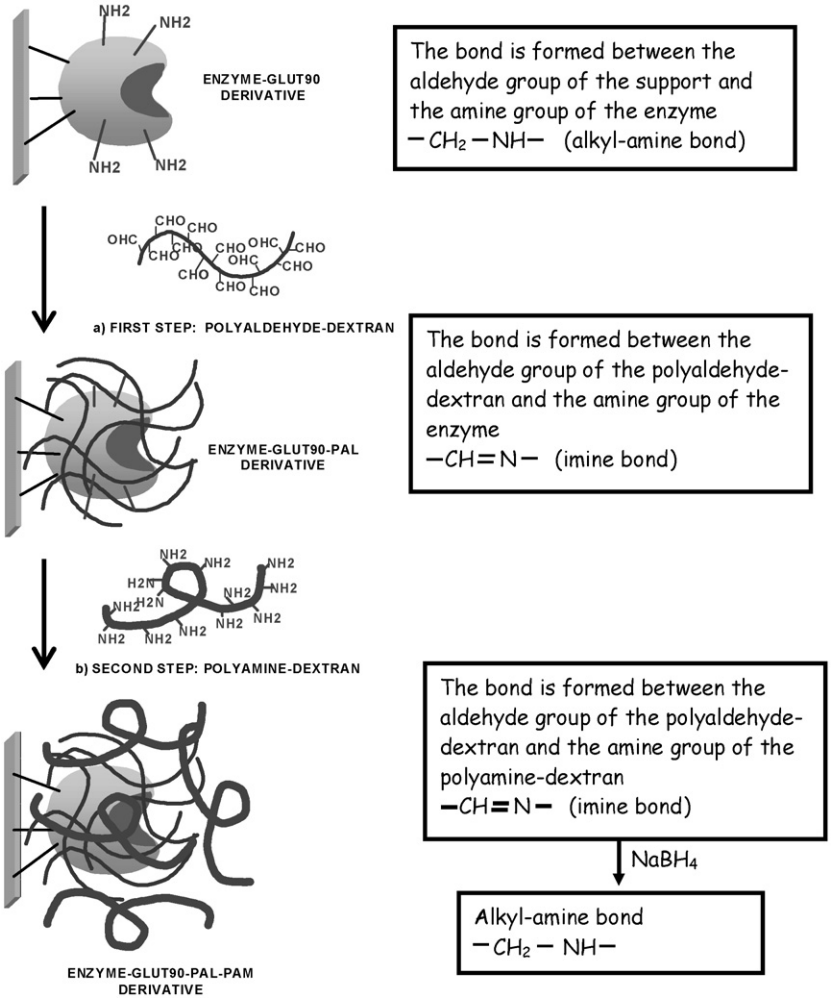
Aliquots of gel suspensions in the appropriate activity buffer were incubated with different concentrations of organic solvents at 30 °C in a total volume of 2 ml (containing a final enzymatic activity of 9 EU/ml for the *E. coli* derivatives, 13 EU/ml for the *K. lactis* derivatives and 25 EU/ml for the *A. oryzae* derivatives). At intervals, samples were taken for activity determination and the residual activity was plotted against time of exposure to the solvent. Solutions of free enzymes containing equivalent amount of EU/ml were treated in the same way. The solvents used were ethanol and acetone in two co-solvent concentrations: 18% (v/v) (3.1 M ethanol; 2.4 M acetone) and 36% (v/v) (6.2 M ethanol; 4.9 M acetone) for both the *E. coli* and *K. lactis* enzyme derivatives. For the *A. oryzae* enzyme derivatives the decay of activity at the lower concentration (18%, v/v) of the co-solvents was not studied because no reduction in the *A. oryzae* enzyme activity had been detected after at least 10 days' storage in these conditions. Instead, *A. oryzae* enzyme derivatives were tested in co-solvent concentrations of 36%, 50% (8.6 M ethanol; 6.8 M acetone) and 75% (v/v) (10.2 M acetone).

2.12. Determination of kinetic parameters (K_M and V_{max}) for A. oryzae enzyme derivatives

Kinetic parameters were determined using varying concentrations of ONPG (0.10–40 mM) in the appropriate activity buffer. The K_M and the V_{max} were determined by the direct linear plot method [32].

2.13. Temperature stability

Aliquots of 2 ml of gel suspension in the appropriate activity buffer containing a final enzyme activity of 9 EU/ml for the



Scheme 1. Modification of the immobilized enzyme with two-step hydrophilization treatment: (a) first step: with polyaldehyde-dextran polymers, and (b) second step: with polyamine-dextran polymer.

E. coli derivatives, 13 EU/ml for the *K. lactis* derivatives and 25 EU/ml for the *A. oryzae* derivatives, were incubated at 53 °C, 45 °C and 60 °C for the *E. coli*, *K. lactis* and *A. oryzae* enzymes, respectively, under gentle stirring. Aliquots were taken at regular intervals, brought to room temperature, and the residual activity was determined. The residual activity was plotted against time of exposure to thermal conditions. Soluble enzyme solutions containing equivalents amounts of enzyme activity were treated in the same way.

3. Results

We selected a three model β-galactosidases enzyme system: those from *E. coli* (a bacterium), *K. lactis* (a yeast) and *A. oryzae* (a fungus). The enzymes are tetrameric, dimeric and monomeric, respectively, and share a low percentage of sequence identity: 14.5% between the *E. coli* and *A. oryzae* enzymes, 13.6% between the *K. lactis* and *A. oryzae* β-galactosidases, and 29.6% between the *E. coli* and *K. lactis* enzyme sequences [33]. Their optimum pH and kinetic properties are also very different, as well as their stability in organic co-solvents and at high temperatures [22,34,35,36]. The enzymes were covalently bound to glutaraldehyde-agarose through surface amino groups (Glut90-derivatives), and to create a hydrophilic nano-environment around the protein molecules a two-step treatment was applied (Scheme 1). First we used polyaldehyde dextrans obtained via periodate oxidation of commercial dextrans [31]; these polyfunctional polymers covalently react with primary amino groups of polypeptides. The immobilized derivative from each enzyme was modified with two different molecular weight polyaldehyde dextrans (41 kDa and 71 kDa). Since the performance of both modified derivatives were quite similar we report the results of the better derivative for each enzyme (polyaldehyde of 71 kDa for the *E. coli* and *A. oryzae* enzymes, and polyaldheyde of 41 kDa for the *K. lactis* enzyme). These modified derivatives were named Glut90-Pal.

The second hydrophilization step was attempted with both the *K. lactis* and *A. oryzae* enzymes, for which a second layer of polyamine dextran was applied over the polyaldehyde envelop; so that the primary amine groups of this new polyfunctional polymer covalently react with the aldehyde groups of the first layer (Glut90-Pal-Pam derivatives).

Table 1
Immobilization and hydrophilization of β-galactosidases on glutaraldehyde-agarose

β-Galactosidase source	Immobilized derivative	Gel-bound protein		Gel-bound activity	
		mg/g gel	%[a]	EU/g gel	%[b]
E. coli	Glut90	5.3	83	172	60
	Glut90-Pal	5.3	83	164	56
K. lactis	Glut90	10.6	85	624	52
	Glut90-Pal	10.6	85	206	20
	Glut90-Pal-Pam	10.6	85	67	5
A. oryzae	Glut90	12.2	54	357	38
	Glut90-Pal	12.2	54	289	31
	Glut90-Pal-Pam	12.2	54	207	22

[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).

3.1. Immobilization of β-galactosidase

The results of the protein content and activity yields after immobilization for the three enzymes are shown in Table 1. The hydrophilization treatment had only a minor negative effect on the immobilized activity of the *E. coli* and *A. oryzae* enzyme derivatives as compared to the strong inactivating effect of the immobilization process on to Glut90-agarose. In the case of the *K. lactis* β-galactosidase derivative both the first and the second hydrophilization treatments had a pronounced effect on enzyme activity, and the second hydrophilization step practically eliminated this activity, as evidenced by the low gel-bound activity of the Glut90-Pal-Pam derivative.

Among other reasons, the lower resistance of the *K. lactis* enzyme to the immobilization and hydrophilization treatments could be due to its notoriously higher density of charged amino acid residues per molecule. This could promote a more extensive reaction both with the matrix and the polymers that would distort the protein structure, thus accounting for the pronounced lost of activity.

The stabilization of the quaternary structure of the immobilized multimeric proteins was analyzed by SDS-PAGE of the supernatant solutions obtained from boiling the enzyme derivatives in the presence of SDS and mercaptoethanol. This treatment releases from the support any protein monomer not covalently bound (directly or indirectly) to the support. It was not possible to detect the release of any subunits after the treatment with the polyaldehyde-polymer, suggesting it was highly effective in cross-linking subunits and preventing their release (not-shown).

3.2. Stability in co-solvent systems

To characterize the effect of the developed nano-environment, enzyme inactivation kinetics in the presence of ethanol and acetone were studied at two different concentrations, and as a baseline control we report the properties of the Glut90 derivatives. The results of the inactivation curves were analyzed according to the two-step deactivation model proposed by Henley and Sadana [37]. The experimental plots of residual activity versus time at a fixed concentration of co-solvent or at a given temperature were adjusted to exponential decays, simple or double, with or without offset with the help of the Enzfitter program.

3.2.1. E. coli β-galactosidase derivatives

In a previous paper [23], we reported that immobilization of the *E. coli* β-galactosidase on to glutaraldehyde agarose (Glut90 derivative) did not prevent protein denaturation when the co-solvent (acetone or ethanol) concentration was increased from 18% to 36% (v/v). In the present study, when the immobilized enzyme molecules were enveloped with the polyaldehyde polymer, the derivative obtained (Glut90-Pal) showed greatly improved enzyme stability in the presence of organic co-solvents (Fig. 1). At low co-solvent concentrations (18%, v/v), for both ethanol and acetone, the stabilizing effect with respect to the unmodified derivative was so strong that it was impossible to

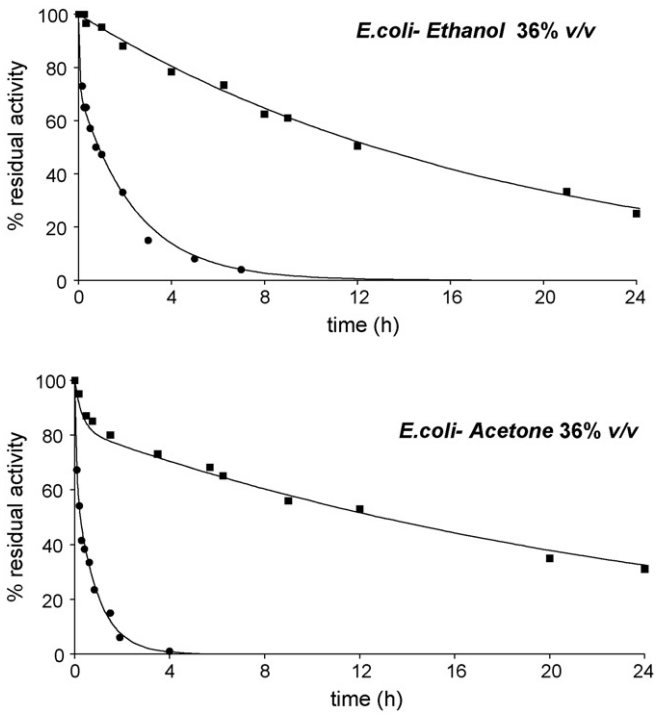


Fig. 1. Stability of the *E. coli* β-galactosidase derivatives in the presence of ethanol and acetone 36% (v/v): (●) Glut90 derivative; (■) Glut90-Pal derivative.

Table 2
Deactivation parameters of β-galactosidase from *E. coli* in co-solvent systems

Co-solvent (%, v/v)	Immobilized derivative	k_1 (h^{-1})	k_2 (h^{-1})	α_1	$t_{1/2}$ (h)
Ethanol, 36%	Glut90	$(2.0 \pm 0.2) \times 10^{1}$	$(4.1 \pm 0.5) \times 10^{-1}$	70 ± 6	0.9
	Glut90-Pal	$(5.4 \pm 0.5) \times 10^{-2}$	0	0	13.0
Acetone, 36%	Glut90	$(1.3 \pm 0.1) \times 10^{1}$	1.1 ± 0.1	58 ± 3	0.2
	Glut90-Pal	3.2 ± 0.2	$(3.9 \pm 0.3) \times 10^{-2}$	81 ± 2	12.8

determine the deactivation constants, since the residual activity remained above 90% for a period of 10 days. When the ethanol concentration was increased to 36% (v/v), the half-life of the modified derivative was improved 14-fold with respect to the Glut90 derivative (Table 2; Fig. 1). In acetone 36% v/v the treatment had a remarkable effect on enzyme stability: the half-life was increased 64-fold.

3.2.2. *K. lactis* β-galactosidase derivatives

The results of kinetic inactivation experiments with *K. lactis* enzyme derivatives in the presence of ethanol (18%, v/v) are shown in Table 3. Due to similar k_1 and α_1 values, the performance of both derivatives, Glut90 and Glut90-Pal, were comparable during the first 60 h of incubation. Afterwards, the modified derivative showed a better profile than the Glut90, owing to its lower k_2 value. When the co-solvent was acetone (18%, v/v), the half-life of the Glut90-Pal derivative was increased 6-fold with respect to Glut90 (Table 3), the stabilization of the Glut90-Pal derivative was due to its higher α_1 value.

Upon increasing the co-solvent concentrations of ethanol or acetone to 36% (v/v) the deactivation profiles of the Glut90-Pal derivative revealed a degree of stabilization (Fig. 2). In ethanol the stabilization was evidenced by the lower k_2 value and higher α_1 which resulted in an increased half-life value of the Glut90-Pal compared to the Glut90 derivative (Table 3). In the presence of acetone 36% (v/v), after 24 h of incubation the Glut90-Pal derivative preserved 20% of the initial activity while the unmodified derivative preserved less than 1%.

3.2.3. *A. oryzae* β-galactosidase derivatives

The β-galactosidase from *A. oryzae* withstood harsher conditions of temperature, acetone and ethanol concentrations than the other two model enzymes. The first hydrophilization treatment

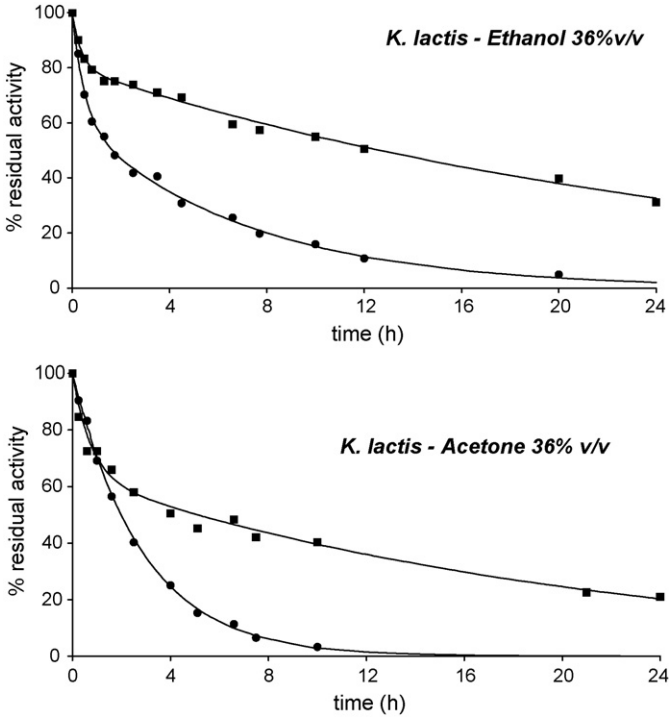


Fig. 2. Stability of the *K. lactis* β-galactosidase derivatives in the presence of ethanol and acetone 36% (v/v): (●) Glut90 derivative; (■) Glut90-Pal derivative.

of the Glut90 derivative resulted in a high degree of stabilization in the presence of 36% (v/v) of both ethanol (11-fold increase in half-life) and acetone (13-fold increase in half-life) (Table 4). When the ethanol concentration was increased to 50% (v/v), the stability of the Glut90-Pal derivative was increased by a factor of 4 (Table 4). However, this derivative in the presence of 50% (v/v) acetone did not show improved stability, indicating that the hydrophilic shell surrounding the enzyme molecule afforded insufficient protection against this high acetone concentration.

Table 3
Deactivation parameters of β-galactosidase from *K. lactis* in co-solvent systems

Co-solvent (%, v/v)	Immobilized derivative	k_1 (h^{-1})	k_2 (h^{-1})	α_1	$t_{1/2}$ (h)
Ethanol, 18%	Glut90	$(1.3 \pm 0.2) \times 10^{-1}$	$(5.4 \pm 0.5) \times 10^{-3}$	71 ± 4	99.3
	Glut90-Pal	$(8.0 \pm 0.2) \times 10^{-2}$	$(1.5 \pm 0.1) \times 10^{-3}$	63 ± 5	221.0
Ethanol, 36%	Glut90	2.0 ± 0.2	$(1.4 \pm 0.1) \times 10^{-1}$	57 ± 1	3.0
	Glut90-Pal	2.7 ± 0.4	$(3.7 \pm 0.1) \times 10^{-2}$	79 ± 1	12.6
Acetone, 18%	Glut90	$(1.9 \pm 0.2) \times 10^{-1}$	$(5.6 \pm 0.9) \times 10^{-3}$	52 ± 3	22.6
	Glut90-Pal	1.2 ± 0.1	$(3.8 \pm 0.3) \times 10^{-3}$	85 ± 1	141.0
Acetone, 36%	Glut90	$(2.5 \pm 0.2) \times 10^{-1}$	0	0	2.0
	Glut90-Pal	1.3 ± 0.2	$(4.8 \pm 0.6) \times 10^{-2}$	62 ± 3	5.1

Table 4
Deactivation parameters of β-galactosidase from *A. oryzae* in co-solvent systems

Co-solvent (%, v/v)	Immobilized derivative	k_1 (h^{-1})	k_2 (h^{-1})	α_1	$t_{1/2}$ (h)
Ethanol, 36%	Glut90	$(3.1 \pm 0.2) \times 10^{-2}$	$(3.8 \pm 0.3) \times 10^{-3}$	32 ± 2	38.2
	Glut90-Pal	$(1.4 \pm 0.2) \times 10^{-3}$	0	0	495.1
Ethanol, 50%	Glut90	$(7.9 \pm 0.5) \times 10^{-1}$	$(6.1 \pm 0.4) \times 10^{-2}$	65 ± 3	5.0
	Glut90-Pal	$(3.4 \pm 0.4) \times 10^{-2}$	0	0	21.7
Acetone, 36%	Glut90	$(2.4 \pm 0.2) \times 10^{-2}$	0	18 ± 1	41.0
	Glut90-Pal	$(1.6 \pm 0.1) \times 10^{-3}$	0	0	441.5
Acetone, 50%	Glut90	$(3.1 \pm 0.2) \times 10^{-1}$	$(2.5 \pm 0.2) \times 10^{-2}$	29 ± 1	5.0
	Glut90-Pal	$(9.7 \pm 0.4) \times 10^{-2}$	0	0	7.2
	Glut90-Pal-Pam	$(1.9 \pm 0.1) \times 10^{-1}$	$(2.9 \pm 0.4) \times 10^{-3}$	70 ± 2	123.0
Acetone, 75%	Glut90	4.4 ± 0.4	$(2.4 \pm 0.1) \times 10^{-1}$	53 ± 3	0.7
	Glut90-Pal-Pam	1.5 ± 0.1	$(1.6 \pm 0.1) \times 10^{-2}$	58 ± 1	10.0

So, a subsequent modification of the *A. oryzae* β-galactosidase-Glut90-Pal derivative was attempted, by further hydrophilization of the nano-environment around the enzyme molecule. A second layer was built over the polyaldehyde-dextran layer, using polyamine dextran. The stability of this hyper-hydrophilized derivative was assayed in the presence of acetone 50% (v/v). Fig. 3 shows the significant positive effect of this further hydrophilization, as the half-life of the hyper-hydrophilic derivative was 25 times greater than the Glut90 unmodified derivative (Table 4). The Glut90-Pal-Pam derivative was tested under very challenging conditions (75%, v/v, acetone), and the result was extremely positive: the twice-modified derivative retained 40% of its original activity after 24 h of incubation, as opposed to 0.2% residual activity of the Glut90 derivative at the same time-point (Table 4; Fig. 3). This stabilization is due to a 15-fold decrease in the k_2 value.

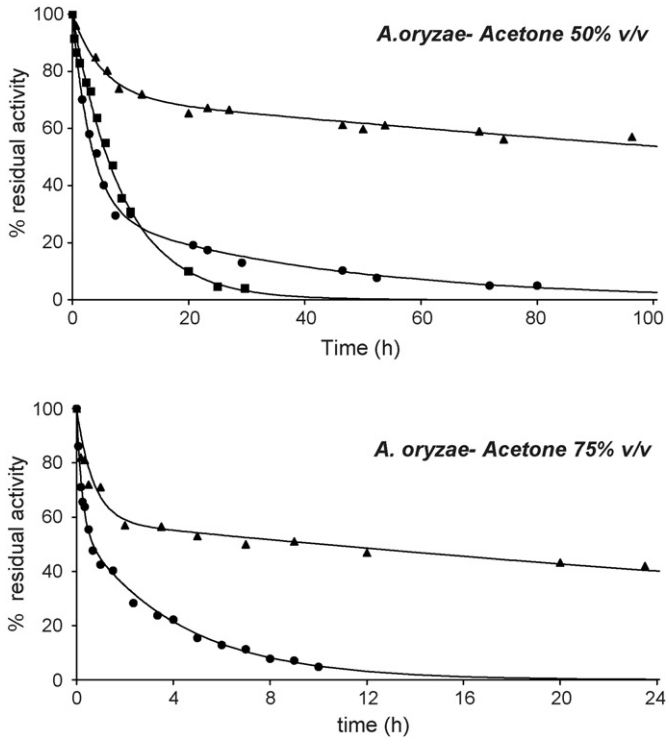


Fig. 3. Stability of the *A. oryzae* β-galactosidase derivatives in the presence of acetone 50% (v/v) and 75% (v/v): (●) Glut90 derivative; (■) Glut90-Pal derivative; (▲) Glut90-Pal-Pam derivative.

3.3. Effect of hydrophilization on the kinetic parameters of the A. oryzae enzyme derivatives

It is a well-known fact that immobilization to solid supports affects the kinetic parameters of enzymes. In the case of the enzyme from *A. oryzae*, immobilization to Glut90-agarose produced an increase in the apparent K_M value from 2.1 mM to 6.3 mM, using ONPG as a substrate. Additionally, the existence of increasing diffusional limitations was evidenced by the higher complexity of the graphical representation of the direct linear plot of the modified derivatives [38]. This reflects either conformational changes that affect the affinity of the enzyme to the substrate and/or the existence of diffusional restrictions due to immobilization. Surprisingly there was not a very important difference in the K_M values between both hydrophilized derivatives, the apparent K_M values of the modified derivatives increased to about 12 and 15 mM ONPG for Glut90-Pal and Glut90-Pal-Pam derivatives, respectively.

3.4. Thermal stability

The effect of the hydrophilization treatment on the thermal stability of the derivatives was variable, but in general it was not a significant improvement, indeed in some cases they performed less well. These single thermal stability experiments may not reflect the true thermodynamic stability of the derivatives, but it has been reported unstabilization effects in similar cases [39,40].

As shown in Table 5, in the case of the *E. coli* enzyme, the hydrophilization treatment had an unfavorable effect on thermal stability. In the case of the *K. lactis* β-galactosidase, the Glut90-Pal derivative showed a slight improvement in the thermal stability profile; its half-life was increased 8-fold, as compared with the unmodified derivative. When the immobilized *A. oryzae* enzyme molecules were surrounded with a single layer of polyaldeheyde dextran, the thermal deactivation perfor-

Table 5
Thermal deactivation parameters from *E. coli*, *K. lactis* and *A. oryzae* derivatives

Enzyme	Immobilized derivative	k_1 (h^{-1})	k_2 (h^{-1})	α_1	$t_{1/2}$ (h)
E. coli (53 °C)	Glut90	$(1.5 \pm 0.2) \times 10^{1}$	$(4.8 \pm 0.3) \times 10^{-1}$	77 ± 4	0.9
	Glut90-Pal	8.0 ± 0.9	$(5.6 \pm 0.3) \times 10^{-1}$	22 ± 2	0.2
K. lactis (45 °C)	Glut90	7.2 ± 0.6	$(1.7 \pm 0.2) \times 10^{-1}$	71 ± 3	2.2
	Glut90-Pal	1.2 ± 0.1	$(8.8 \pm 0.7) \times 10^{-3}$	58 ± 1	17.3
A.oryzae (60 °C)	Glut90	$(3.7 \pm 0.4) \times 10^{-1}$	$(4.4 \pm 0.5) \times 10^{-2}$	17 ± 1	2.5
	Glut90-Pal	$(1.2 \pm 0.2) \times 10^{-1}$	0	0	6.0
	Glut90-Pal-Pam	$(9.9 \pm 0.9) \times 10^{-1}$	$(5.7 \pm 0.7) \times 10^{-2}$	45 ± 4	2.0

mance was improved. However, after further hydrophilization, the Glut90-Pal-Pam derivative showed a decrease in thermal stability with respect to the corresponding stability of the Glut90-Pal derivative; its deactivation profile was similar to the unmodified derivative (Glut90).

The poor thermal stability observed for our hydrophilized derivatives is in agreement with previous reports [39,40].

4. Discussion

The immobilization of the β-galactosidases on glutaraldehyde-activated supports is an established method that proves to serve as a first step of stabilization, against both temperature and low concentrations of organic co-solvents [22,23]. In general, this technique preserved a high percentage of enzyme activity bound to the support (Table 1). However, the enzyme derivatives did not remain active in the presence of high concentrations of organic co-solvents. In order for them to withstand these more demanding conditions, it was necessary to find a strategy to reinforce the stability of the immobilized enzyme. We attempted to generate a hydrophilic environment surrounding the immobilized enzyme molecules, as a complementary strategy to prevent interaction between the organic solvent molecules and the enzyme surface. The modification with the polyaldehyde polymers had at least two different consequences on the immobilized enzyme molecules. Firstly, in the case of the oligomeric enzymes, cross-linking between the monomers took place, as demonstrated by the absence of protein released by boiling, reduction and SDS treatment of the modified derivatives. The second consequence was a dramatic stabilization of the derivatives when they were exposed to high concentrations of organic co-solvents evidencing the hydrophilization of the enzyme nano-environment (Tables 2 and 3).

The β-galactosidase from *A. oryzae* has a very good transglycosylation activity [27], it is the most stable of the three enzymes studied, and it appears to be the most promising enzyme for catalyzing synthetic reactions in high co-solvent concentrations. Noteworthy, the Glut90-Pal-Pam derivative showed a high degree of stabilization in the presence of a high concentration of co-solvents such as 50% (v/v) acetone, and even in more demanding conditions (75%, v/v, acetone) (Table 4 and Fig. 3), which is promising for biotechnological applications. The stabilization due to hydrophilization achieved in this work is comparable to the effect observed by Wilson et al. for the dextran sulfate and polyethyleneimine-coated CLEAs [40].

Even though the increase in K_M due to hydrophilization may be evidence of certain diffusional restrictions, the hydrophilic shell cannot be considered a very closed structure preventing the transfer of substrates and products between the bulk solution and the enzyme active centers. The hydrophilic shell appears to behave as an open structure that could hinder the access of co-solvent molecules to the enzyme surface. This can be understood as a "partition effect" that explains, at least in part, the observed hyper-stabilization.

The poor thermo-stabilization by hydrophilization suggests that the possible cross-linking of the enzyme by the polymers had only a marginal effect on the rigidity of the enzyme. If the rigidity of the enzyme had actually been increased, a parallel increment in the thermal stability of the enzyme should have been observed. Thus, hydrophilization appears especially effective in preventing the unfolding of the hydrophobic core which occurs when the protein is exposed to a medium of lower polarity, as in the presence of co-solvents.

The three model enzymes we studied clearly show that stabilization achieved under very drastic polar co-solvent concentration conditions is directly related to the hydrophilization of the nano-environment, thus showing the enormous potential of hydrophilization as a strategy for stabilizing enzymes. The limit of the strategy appears to be the stability of the immobilized enzyme in the conditions required for the hydrophilization treatment.

Moreover, since the two-step strategy has also proved successful with a number of other enzymes such as lipases (from *Mucor miehie* and *Candida rugosa*) [25], pig liver esterase [26] and penicillin G acylase [24,39], it appears that the generation of these hyper-hydrophilic nano-environments may provide the basis of a general method for the stabilization of enzymes in the presence of organic co-solvents.

5. Conclusions

The hyper-hydrophilic nano-environment generated around the enzyme molecule with polyaldehyde and polyamine dextran polymers, has shown itself to be a powerful tool for the stabilization of enzymes in the presence of high concentrations of co-solvents.

This two-step strategy could, in principle, be used to enhance the stability of any other enzyme in the presence of organic co-solvents.

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