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Effect of increasing co-solvent concentration on the stability of soluble and immobilized β-galactosidase

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Abstract

The effect of increasing concentration of co-solvents on the stability of both soluble and immobilized *E. coli* β-galactosidase was studied. The enzyme was immobilized on to glutaraldehyde-agarose and the co-solvents tested were: *N*,*N*-dimethylformamide (DMF), ethanol, acetone, and dioxane (6–36% v/v). Deactivation kinetics were analyzed according to the two-step deactivation model proposed by Henley and Sadana. A multi-temperature study revealed that the immobilized derivative is considerably more stable than the soluble enzyme, even at high temperature where the half life of the soluble enzyme is less than 1 h.

Enzyme immobilization did achieve thermal and solvent stabilization at low concentrations, but this task proved more difficult with increasing co-solvent concentrations. In the case of ethanol and acetone, with the increase in co-solvent concentration, the immobilized derivative became less stable than the soluble enzyme. Immobilization may impose certain constraints on the protein structure which have the effect of sensitizing it to denaturation at high co-solvent concentrations. Complementary stabilization strategies are, therefore, being studied.

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1. Introduction

Chemical synthesis of carbohydrates and glycoconjugates requires skilful and selective protection and deprotection reactions. In contrast, enzymatic processes can provide regio- and stereo-selective products in one-step under mild conditions [1,2]. However, due to their complex molecular structure, biocatalysts are intrinsically unstable. Many applications demand the use of enzymes in reaction mixtures containing organic co-solvents that challenge the stability of the biocatalysts [3]. Enzyme stability is thus a key parameter that limits their application in industry [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].

Glutaraldehyde-based chemistry is a very traditional and effective method for immobilization and stabilization [7,8]. In this paper, we report the effect of immobilization on the stability of the *E. coli* β-galactosidase with increasing co-solvent concentrations, and with increasing temperature.

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2. Methods

2.1. Synthesis of glutaraldehyde-agarose

Glutaraldehyde-agarose containing 90 μmol of glutaraldehyde per gram of suction dried gel was prepared as described previously by Guisán et al. [7].

2.2. Protein assay

Protein content of *E. coli* β-galactosidase (EC 3.2.1.23, grade VI, Sigma) was determined by measuring the absorbance value at 280 nm ($\varepsilon^{1\,\mathrm{mg/ml}} = 2.09$).

2.3. Enzyme activity

The activity of β-galactosidase was assayed at room temperature, using as substrate 10 mM *o*-nitrophenyl-β-D-galactopyranoside (ONPG, Sigma) in 50 mM sodium phosphate buffer, pH 7.5, containing 3 mM $MgCl_2$ (activity buffer). 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.

2.4. Immobilization of β-galactosidase onto glutaraldehyde-agarose

This was performed as described by Giacomini et al. [8].

2.5. Stability experiments

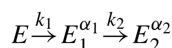
Aliquots of 2 ml of gel suspensions in activity buffer, and of soluble enzyme, each containing a final concentration of 9 EU/ml, were incubated for 6 h at different temperatures (48, 50, 53, and 55 °C) or for 7 days with different buffer–organic solvent mixtures, under gentle stirring. Aliquots were taken at regular intervals and the residual activity was determined. The co-solvents assayed were: ethanol, acetone, dioxane and dimethylformamide (6, 18 and 36% v/v). The experiments were performed in triplicate.

3. Results and discussion

β-Galactosidase from *E. coli* was immobilized on to glutaraldehyde activated agarose. We studied the effect of increasing co-solvent concentration on the stability of the soluble and immobilized enzyme. These results were compared with those for the effect of increasing temperature on the stability of soluble and immobilized enzyme.

3.1. Multi-temperature evaluation of the stability of soluble and immobilized enzyme

Thermal stability studies for both soluble and immobilized enzyme were performed at 48, 50, 53, and 55 °C. The thermal deactivation curves for the soluble and immobilized enzyme derivatives were analyzed according to the two-step deactivation model proposed by Henley and Sadana [9,10]:



$$E \xrightarrow{k_1} E_1^{\alpha_1} \xrightarrow{k_2} E_2^{\alpha_2}$$

The kinetics of enzyme inactivation can be described assuming the existence of partially deactivated states (E_1 and E_2) with nonzero specific activity, where k_1 and k_2 are first order deactivation velocity coefficients. A weighted-average activity expression has been derived by the same authors for a nonzero specific activity for the final state E_2:

$$A = \left[100 + \left(\frac{\alpha_1 k_1}{k_2 - k_1}\right) - \left(\frac{\alpha_2 k_2}{k_2 - k_1}\right)\right] \exp(-k_1 t) - \left(\frac{k_1}{k_2 - k_1}\right)(\alpha_1 - \alpha_2)\exp(-k_2 t) + \alpha_2$$

where $\alpha_1 = E\,[(E_1/E) \times 100]$ and $\alpha_2 = E\,[(E_2/E) \times 100]$. The experimental plots of residual activity versus time at a fixed temperature were adjusted to exponential decays, simple or double, with or without offset, with the help of the Enzfitter program; and the parameters: $k_1, k_2, \alpha_1, \alpha_2$ and the half life of the biocatalysts were calculated. We defined enhanced enzyme stability, in this paper, as a decrease in the deactivation parameters k_1, k_2 (and a corresponding increase in half life) or as an increase in enzyme residual activity α_1.

There was a good fit between our experimental inactivation kinetics and a double exponential decay model with significant values of the constant k_2, so the enhanced stabilization is shown by the increase in half life. At temperatures ranging from 48 to 53 °C, the half life of the immobilized derivative was increased about three-fold with respect to the corresponding soluble enzyme (Fig. 1). At the highest temperature

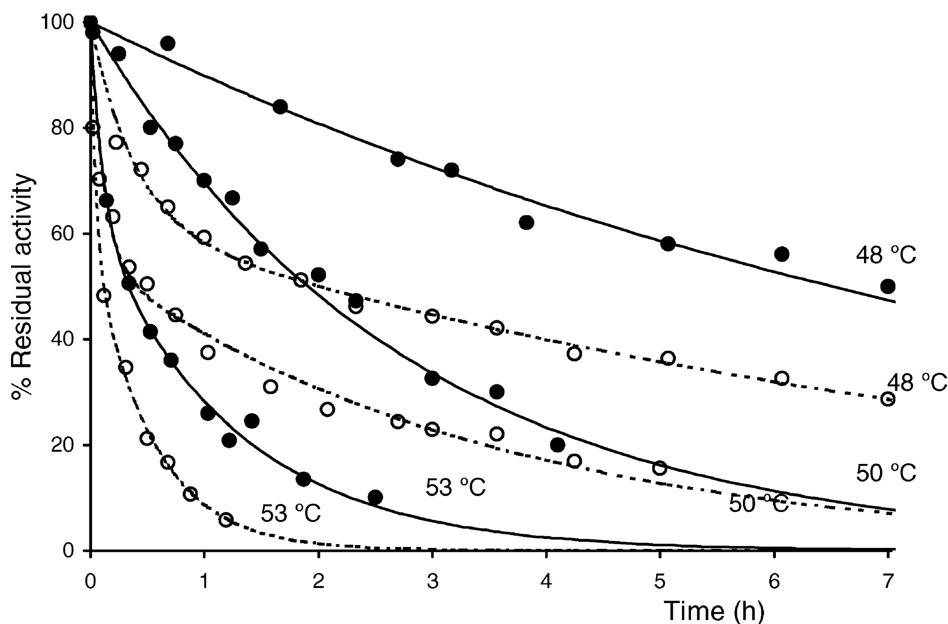


Fig. 1. Multi-temperature evaluation of the stability of soluble and immobilized β-galactosidase. (○): Soluble β-galactosidase, (●): β-galactosidase-glutaraldehyde-agarose.

studied (55 °C), the derivative was also more stable than the soluble enzyme, but the stabilization factor was reduced to 1.8.

3.2. Solvent stability

The soluble enzyme was studied at 6, 18 and 36% v/v of ethanol, acetone, DMF and dioxane. The enzyme derivative was incubated in the following co-solvent mixtures: 6 and 18% v/v for dioxane, and 18 and 36% v/v for ethanol, acetone and DMF. The co-solvent concentrations were chosen so that the half life of the soluble enzyme was at least 40 h for the lowest concentration studied. Thus, for dioxane the co-solvent concentrations chosen were 6 and 18% v/v.

At the lowest co-solvent concentration, in each case, the derivative was always more stable than the soluble enzyme. In the case of ethanol 18% v/v, the derivative showed a very stable state E_1 with a high residual activity of 73%, while the soluble enzyme had a double exponential decay without offset (Fig. 2 and Table 1). The incubation with acetone 18% v/v showed that the soluble enzyme had a stable state E_1 with an α_1 value of 76%. The derivative followed a model of a simple exponential decay with offset, in which the k_1 value was one order of magnitude lower than the k_1 of the soluble enzyme. The derivative was more stable than the soluble β-galactosidase, at least for the first 20 h of incubation. In dioxane 6% v/v, the half life of the immobilized derivative is 10-fold greater than that of the soluble enzyme. The denaturing effect of DMF 18% v/v is similar to that of acetone 18% v/v, and again in this case the derivative is more stable than the soluble β-galactosidase, during the first 20 h of incubation.

With higher co-solvent concentrations, the immobilized derivative becomes less stable than the soluble enzyme in the presence of some co-solvents such as 36% v/v acetone and 36% v/v ethanol. This is clearly shown by the corresponding decrease in half life of the immobilized derivative with respect to the soluble form (Fig. 2 and Table 1). In the other cases, such as 18% v/v dioxane, the half life is increased from 8 h for the soluble enzyme to 100 h for the derivative and in 36% DMF, the half life is increased from 0.3 h for the soluble enzyme to 1 h for the derivative. Thus, the effect of immobilization on enzyme stability at high co-solvent concentrations depends on the properties of the co-solvent.

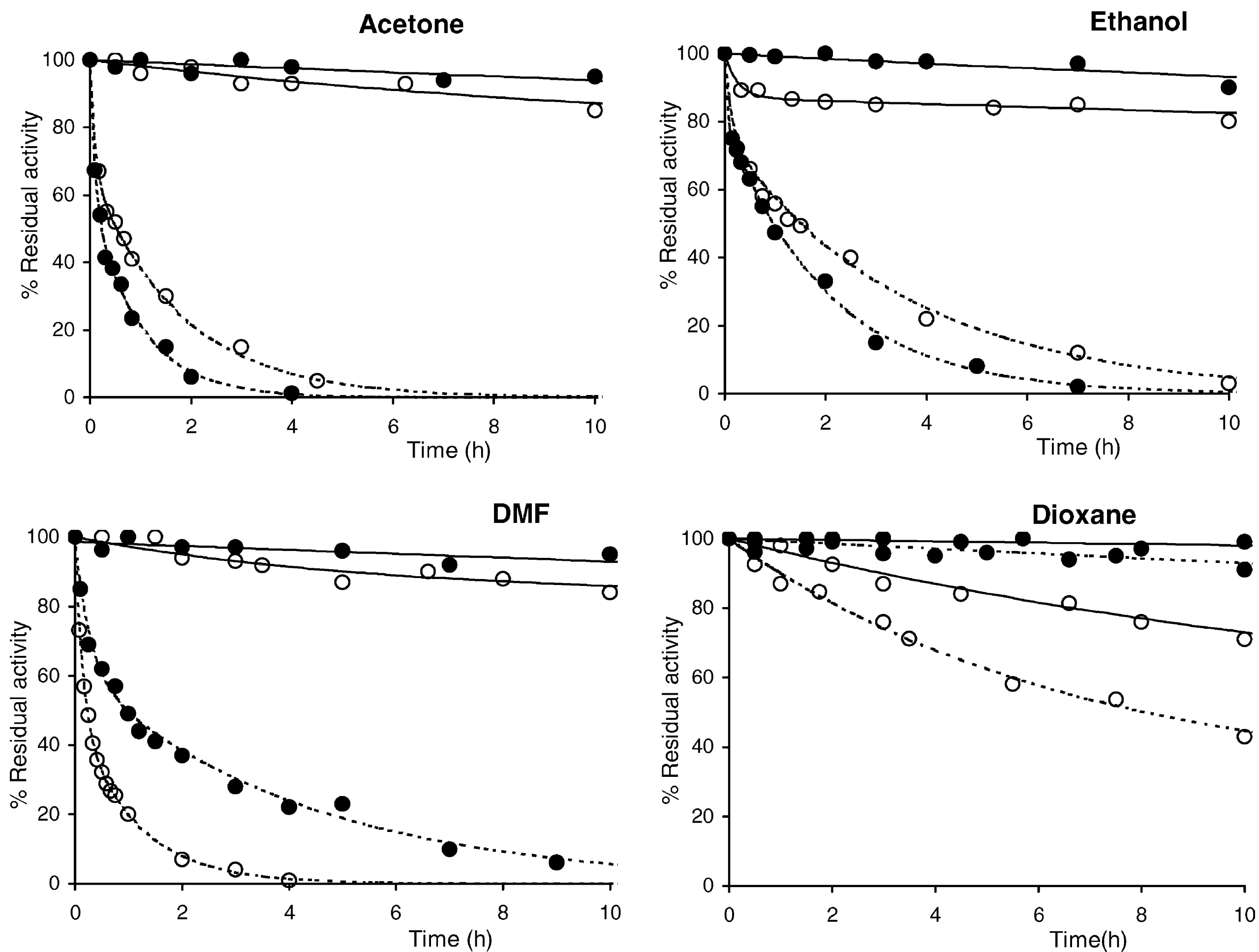


Fig. 2. Stability of soluble and immobilized β-galactosidase in co-solvent systems. (○): Soluble β-galactosidase, (●): β-galactosidase-glutaraldehyde-agarose, (—): acetone, ethanol and DMF 18% v/v, dioxane 6% v/v, (- - -): acetone, ethanol and DMF 36% v/v, dioxane 18% v/v.

Table 1
Deactivation parameters of β-galactosidase in co-solvent systems

Co-solvent	Enzyme	k_1 (h^{-1})	k_2 (h^{-1})	α_1	$t_{1/2}$ (h)
Ethanol 18%	Soluble	5.2	8.2×10^{-3}	87	55
	Immobilized	3.2×10^{-2}	0	73	–
Ethanol 36%	Soluble	9.2	2.1×10^{-1}	74	1.9
	Immobilized	$2.0 \times 10^{+1}$	4.1×10^{-1}	70	0.9
Acetone 18%	Soluble	6.7×10^{-2}	0	76	–
	Immobilized	8.0×10^{-3}	0	22	130
Acetone 36%	Soluble	8.1	5.3×10^{-1}	63	0.6
	Immobilized	$1.3 \times 10^{+1}$	1.1	58	0.2
Dioxane 6%	Soluble	6.6×10^{-2}	0	43	38
	Immobilized	1.7×10^{-3}	0	0	410
Dioxane 18%	Soluble	1.4×10^{-1}	0	27	8
	Immobilized	7.1×10^{-3}	0	0	100
DMF 18%	Soluble	3.4×10^{-1}	0	77	–
	Immobilized	4.7×10^{-3}	0	0	150
DMF 36%	Soluble	7.0	6.9×10^{-1}	46	0.3
	Immobilized	3.2	2.3×10^{-1}	56	1.0

In conclusion, immobilization of the enzyme achieved thermal and solvent stabilization at low concentrations, but enhancing stability proved to be a difficult task with increasing co-solvent concentrations in some cases. The different behaviors, in terms of stability in certain thermal and solvent conditions, of soluble and immobilized enzyme, may arise from the different mechanisms of inactivation when temperature and solvent conditions are changed. Glutaraldehyde is known to stabilize enzymes by rigidification of the protein structure, but it seems to be less able to prevent unfolding at high concentrations of organic co-solvents. Immobilization may impose certain constraints on the protein structure which have the effect of sensitizing it to denaturation at high co-solvent concentrations. We are therefore undertaking further studies of complementary stabilization strategies.

Acknowledgements

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