Journal of Molecular Catalysis B: Enzymatic 65 (2010) 91–101

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Improvement of activity and stability of soluble and sol–gel immobilized naringinase in co-solvent systems

Helder Vila-Real, António J. Alfaia, António R. Calado, Maria H.L. Ribeiro *

Faculty of Pharmacy, Research Institute for Medicines and Pharmaceutical Sciences (i-Med-UL), University of Lisbon, Av. Prof. Gama Pinto, 1649-003 Lisbon, Portugal

ARTICLE INFO

Article history:
Available online 1 February 2010

Keywords:
Naringin
Co-solvent
Naringinase
Sol–gel (TMOS + glycerol)
Stability
Deactivation kinetics

ABSTRACT

The hydrolysis of naringin, a bitter flavonone glycoside, with naringinase leads to reducing sugars (rhamnose and glucose), to prunin and to the aglycone, naringenin. To overcome the low solubility of naringin in the enzymatic reaction media, the effect of different solvents was studied, in order to improve the productivity and yield of the system. The effect of increasing concentration of co-solvents on the stability of both soluble and immobilized naringinase expressing α-L-rhamnosidase and β-D-glucosidase activities was evaluated. The enzyme was immobilized onto sol–gel matrices of tetramethoxysilane and glycerol. Combining the higher naringin solubility, and the higher residual activity of both α-L-rhamnosidase and β-D-glucosidase expressed by naringinase, eight solvents were chosen for stability and activity studies: dimethyl sulfoxide, *N*,*N*-dimethylmethanamide, methanol, ethanol, acetone, tetrahydrofurane, 1,2-dimethoxyethane and 1,4-dioxane.

Deactivation of soluble naringinase was analyzed according to a first-order kinetic model. For the sol–gel immobilized enzyme the two-step deactivation model, of Henley and Sadana, was adjusted.

Sol–gel immobilization stables naringinase in all tested co-solvents systems. This effect was specially pronounced at higher co-solvent concentration (10%). The half-life of α-L-rhamnosidase and β-D-glucosidase expressed by naringinase even increased 21- and 59-fold, respectively, in aqueous co-solvation with tetrahydrofurane.

These are high innovative and sounding results showing the protective effect of immobilization onto sol–gel (tetramethoxysilane + glycerol) matrices with naringinase in co-solvent systems, which is a great advantage for non-conventional biocatalysis.

1. Introduction

Numerous applications of enzymes on glycosides hydrolysis/synthesis demand the use of co-solvents mainly due to the low solubility of substrates/products. Among glycosides, flavonoids are an important group of compounds possessing biologic properties such as antioxidant, anti-inflammatory or neuroprotective. However some of them present low aqueous solubility which restrains its production with high yields. Naringin is an example of a flavonoid showing low water solubility and naringinase is an enzyme often used on its hydrolysis. Moreover, high substrate concentrations are required to achieve high reaction rates, and those concentrations largely exceed the solubility of most non-polar substrates in water. One approach used to increase substrate solubility is medium engineering involving the substitution of aqueous reaction media by non-conventional media.

Enzymes usually require aqueous environments in which the organic substrates are poorly soluble and in some cases even unstable. A straightforward solution to this problem is the application of biphasic or co-solvent systems [1]. In biphasic systems, the enzyme is dissolved in the aqueous phase, while the hydrophobic substrate is present in a high concentration in the organic phase. As the occurrence of a liquid–liquid interface and presence of residual amounts of organic solvent in water can lead to deactivation of the biocatalyst. Different approaches to guide the choice of the proper solvent have been proposed, such as that reported by Laane [2,3] who suggested the use of the polarity of solvents, expressed by the log *P* value (logarithm of the partition coefficient of a given compound in the standard n-octanol/water two-phase system) as the main criterion for optimizing organic solvents in multi liquid-phase biocatalysis.

The addition of water-miscible solvents like acetone, ethanol, acetonitrile or dioxane has often been used to increase the solubility of apolar reactants. Usually, the addition of small amounts of a water-miscible solvent has little effect on the biocatalyst activity and stability, in some cases the addition of low concentrations of these solvent may even result in an enhanced enzyme activity and

* Corresponding author. Tel.: +351 21 7946400; fax: +351 21 7946470.
E-mail address: mhribeiro@ff.ul.pt (M.H.L. Ribeiro).

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doi:10.1016/j.molcatb.2010.01.024

stability. However, an increase in the concentration of most water-miscible solvents may have an inhibitory effect on the biocatalyst [4].

The resulting reaction mixtures containing organic (co-)solvents may challenge the stability of the biocatalysts. In fact, enzyme stability is a key parameter that limits their application in industry. Several approaches have been developed to improve enzymes activity, stability, and re-usage capacity. Such improvements have been achieved by chemical, physical, or modification of the native enzyme. Selection of enzymes from thermophilic organisms, use of stabilizing additives, modification of native enzyme using different techniques, like site directed mutagenesis have been addressed.

Biocatalysts immobilization using different methods, like crosslinking with bifunctional reagents, entrapment, are among the most used strategies to improve enzyme stability [5–7].

Among these methods, sol–gel is a very effective process for the immobilization and stabilization of enzymes in reaction media. The mild conditions of this inorganic polymerization method allow the association of organic systems within mineral phases. Some advantages, such as materials optical transparency, possibility of chemical modifications and pore size tuning, made this method a very convenient one [8,9].

Organic biosynthesis is a wide application field for enzyme immobilized in sol–gel materials, including biocatalysts that allow the occurrence of sequential reactions in a restricted place [9].

Naringinase used in the enzymatic hydrolysis of glycosides was immobilized in sol–gel matrices and studied for its standard reaction (naringin hydrolysis). In this reaction, naringinase, which expresses α-L-rhamnosidase and β-D-glucosidase activities, leads first to prunin and rhamnose and after to the aglycone naringenin and glucose (Fig. 1) [10]. The glycosidic residue is important for the biologic activity of the glycosides, which can be improved through enzymatic deglycosylation [11,12]. Naringenin is reported to be antioxidant, antiulcer and anti-inflammatory [11,12], inducing apoptosis through the activation of caspase-3 cascade [13] and preventing neurodegenerative diseases such as Alzheimer's [14].

To discriminate between the two enzymatic activities expressed by naringinase enzyme complex, the use of specific substrates is of major significance. With this purpose, *p*-nitrophenyl α-L-rhamnopyranoside and *p*-nitrophenyl β-D-glucopyranoside were used as specific substrates for α-L-rhamnosidase and β-D-glucosidase, respectively. So, both enzymatic activities of naringinase can be followed and easily measured without the need of a previous subunit protein separation and purifying procedures. The main goals of this work were (i) to test different co-solvents to overcome the low solubility of naringin in aqueous system, (ii) to study the effect of the different co-solvents with increasing concentrations on the activity and stability of β-D-glucosidase and α-L-rhamnosidase activities expressed by soluble and sol–gel immobilized naringinase and (iii) to model β-D-glucosidase and α-L-rhamnosidase deactivation kinetics and, finally, (iv) to study naringin hydrolysis with naringinase in aqueous co-solvent systems.

2. Material and methods

2.1. Chemicals

Naringin, *p*-nitrophenyl α-L-rhamnopyranoside, *p*-nitrophenyl β-D-glucopyranoside were from Sigma–Aldrich and tetramethoxysilane (TMOS) from Fluka. 3,5-Dinitrosalicylic acid (DNS), glucose were from Merck while the protein assay dye reagent concentrate (cat. no. 500-0006) was from Bio-Rad. All other chemicals were analytical grade and obtained from various sources.

2.2. Enzyme stock solution

Naringinase (CAS no. 9068-31-9, cat. no. 1385) from *Penicilliun decumbens* was obtained from Sigma–Aldrich and stored at −20 °C. The lyophilized naringinase powder was dissolved in 20.0 mM acetate buffer pH 4.0, at 625 g L^{-1} concentration. This enzyme stock solution was kept at 4 °C for a month.

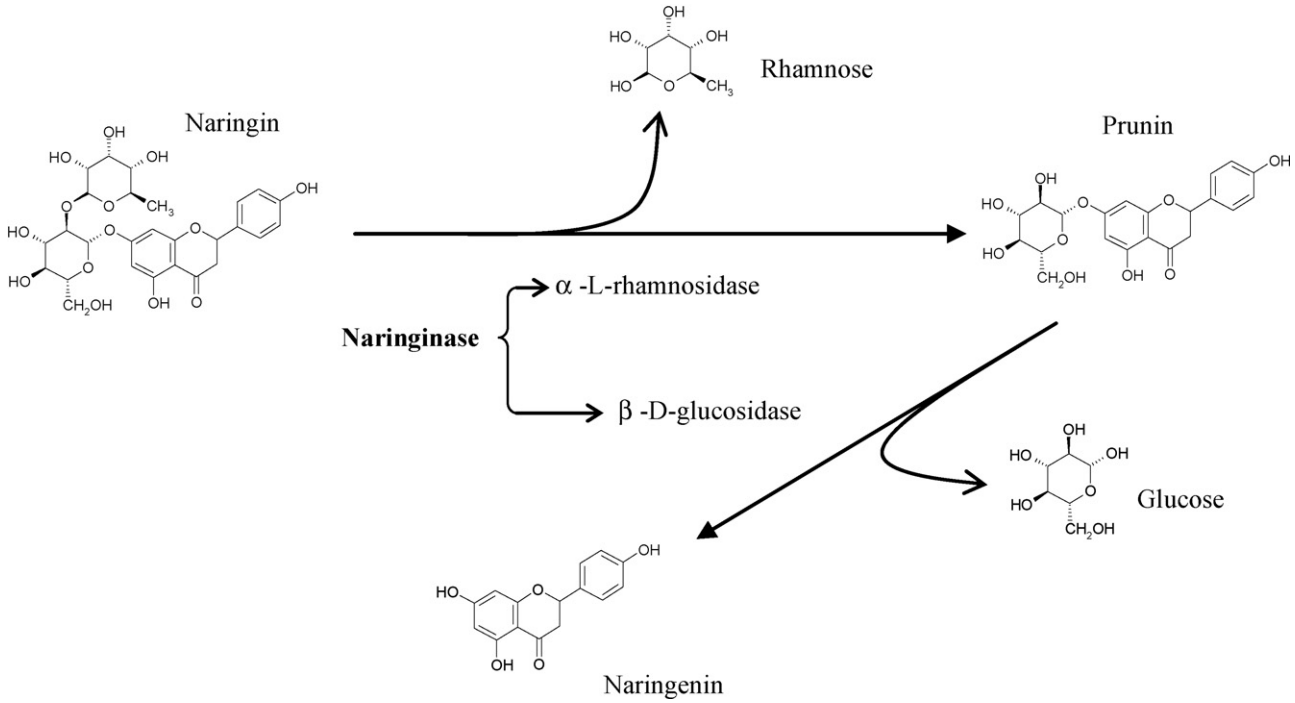

Fig. 1. Hydrolysis of naringin into prunin, rhamnose, glucose and naringenin by naringinase expressing α-L-rhamnosidase and β-D-glucosidase activities.

2.3. Analytical methods

Reducing sugars were determined using DNS microassay. In this work the DNS macroassay [15] was modified into a microassay procedure using a microtiter plate. The advantages of this microassay are higher repeatability, quickness, large sample analysis number as well as sample volume reduction. The microassay developed by us consists of the addiction of DNS reagent (85 μL) to equal sample volume in a 96-microtiter plate. The microplate was heated during 5 min, at 100 °C and then cooled down in a water bath at room temperature. Absorbance was read at 575 nm. The reducing sugars concentration was determined against a glucose calibration curve.

The protein content was determined using Bradford assay from Bio-Rad protein microassay procedure, using a naringinase calibration curve [16].

The concentration of the flavanones, naringin and naringenin, was determined through the absorbance measured at 280 nm, using a calibration curve of each flavanone.

The concentration of *p*-nitrophenyl α-L-rhamnopyranoside and *p*-nitrophenyl β-D-glucopyranoside was evaluated at 340 nm, using a calibration curve of each compound.

2.4. Naringin solubility

Due to the low solubility of naringin in water several solvents were tested in order to increase substrate solubility. The solvents were chosen according to their log*P* and dielectric constant. A naringin solubility test was assessed trough the complete dissolution of 10 μmol of naringin in 50, 100, 200, 400 and 800 μL of the pure solvent at 25 °C.

The following solvents were tested with the aim of use in biphasic system: octane and n-hexane, cyclohexane, 1-octanol, 2-octanol, 1-hexanol, toluene, anisole, ethylic ether, ethyl acetate, carbon tetrachloride, chloroform.

In co-solvent systems the following solvents were tested: propylene carbonate, dimethyl sulfoxide, *N*,*N*-dimethylacetamide sulfoxide *N*,*N*-dimethylmethanamide, *N*,*N*-dimethylacetamide, methanol, acetonitrile, ethanol, acetone, 1-propanol, 2-propanol, 1-butanol, 2-butanol, 3-methyl-1-butanol, 2-butoxyethanol, tetrahydrofurane, 1,2-dimethoxyethane, 2-methyl-2-butanol, 1,4-dioxane.

2.5. Immobilization protocol

Naringinase immobilization was preformed on sol–gel matrices with lens shape. The silica sol was prepared in an eppendorf tube (1.5 mL) by mixing glycerol (96 mg), distilled water (70 μL), 40 mM HCl solution (15 μL) and tetramethoxysilane (TMOS) (300 μL). This mixture was sonicated at 0 °C during 20 min, after which one layer was formed. The hydrogel was prepared in a 96-well plate by mixing the sol (25 μL) with an enzyme solution of 250 g L^{-1} (25 μL). The sol–gel was mixed in a vortex and left in an opened 96-well plate, at room temperature for 14 h. Afterwards the biocatalyst was used in bioconversion trials.

2.6. Enzyme activity

The activity of α-L-rhamnosidase expressed by naringinase enzyme complex was evaluated using the substrate *p*-nitrophenyl α-L-rhamnopyranoside in a concentration of 0.20 mM in acetate buffer (20.0 mM) pH 4.0, while the activity of expressed β-D-glucosidase was determined using the substrate *p*-nitrophenyl β-D-glucopyranoside at 0.20 mM concentration in (20.0 mM) also in acetate buffer pH 4.0. A naringinase concentration of 31.3 mg L^{-1} was used in these experiments.

The enzymatic hydrolysis of the substrates *p*-nitrophenyl α-L-rhamnopyranoside and *p*-nitrophenyl β-D-glucopyranoside with free and immobilized naringinase was followed spectrophotometrically (*Zenith 3100* spectrofluorimeter) at λ = 340 nm. The absorption was measured every 1 min during 30 min, 30.0 °C, at atmospheric pressure. In both reactions 1 mol of substrate led to 1 mol of product. A calibration curve was built for each substrate and respective product. The enzyme activity (*A*) of α-L-rhamnosidase and β-D-glucosidase expressed by naringinase was calculated by linear regression on the first data-points during the initial 30 min reaction time.

Naringin bioconversion studies were carried out in standard solutions of naringin in acetate buffer (20 mM) pH 4.0, at 45 °C, using 250 mg L^{-1} naringinase concentration. The naringin concentration varied from 0.30 to 3.0 mM in the aqueous system and to 30 mM in the aqueous co-solvent systems.

The naringinase activity was calculated by first-order polynomial regression data fit of the initial data, collected every 1 min during 10 min.

The fit of Michaelis–Menten model $\{V = ([S]\cdot V_{max})/([S] + K_M)\}$ $\{V$ is the initial rate (mM min^{-1}) [*S*] the substrate concentration (mM), V_{max} the maximum initial rate (mM min^{-1}), K_M is the Michaelis–Menten constant (mM)$\}$ to experimental data was carried out through non-linear regression by minimising the residual sum of squares between the experimental data-points of the initial rate *vs* substrate concentration and those estimated by the model, using Solver add-in from Microsoft Excel 2003 for Windows XP, considering the following options: Newton method; 100 iterations, precision of 10^{-6}, 5% of tolerance and 1×10^{-4} convergence. The kinetic parameters estimated by linear regression using the Lineweaver-Burk plot, which results from the linearization of the Michaelis–Menten equation: $\{1/V = 1/V_{max} + (K_M/V_{max})\,(1/[S])\}$, were used as initial values of the non-linear regression parameters. The non-linear regression parameters were constricted to positive numbers.

2.7. Stability experiments

The activity and stability of α-L-rhamnosidase and β-D-glucosidase expressed by soluble and sol–gel immobilized naringinase were evaluated, in several aqueous co-solvent systems using increased solvent concentrations.

Firstly, a deactivation protocol was established in order to study the selective stability of α-L-rhamnosidase and β-D-glucosidase expressed by naringinase enzyme complex. Enzyme activity deactivation assays were followed in 20 mM acetate buffer at pH 4.0, using 0%, 2.5%, 5% and 10% (v/v) of co-solvent, at 65 and 80 °C, respectively for β-D-glucosidase and α-L-rhamnosidase of naringinase used soluble and immobilized on sol–gel matrices. In the first case an enzyme concentration of 31.3 mg L^{-1} were used in 20 mM acetate buffer at pH 4.0, with 5% (v/v) of co-solvent, during 30 min at 65 °C and 80 °C, respectively, and aliquots were collected during 4 h. The activity of non-deactivated soluble naringinase (A_0) was used as the reference (100% activity). A primary solvent screening according to the effect over α-L-rhamnosidase and β-D-glucosidase expressed by free naringinase (625 mg L^{-1}) was carried out.

In the second case, the sol–gel immobilized naringinase (31.3 mg L^{-1}) was used for periods of 30 min under the above mentioned deactivation conditions during a total of 6 h. After each deactivation period the matrices were washed and its activity was measured. The enzyme activity (A_0) of freshly prepared matrices before the first deactivation run was defined as 100%.

To describe the deactivation kinetics, each experimental run, was converted to the fraction of the original activity, i.e., its residual activity. This residual activity, A_r ($A\cdot A_0^{-1}$), was defined as the ratio

between the specific activity after each run (A) and the specific activity of the first run (A_0).

The deactivation rate constants, according to the model adjusted, were determined through non-linear regression by minimising the residual sum of squares between the experimental data points of the residual activity *vs* runs and those estimated by the model, using Solver add-in from Microsoft Excel 2003 for Windows XP, considering the following options: Newton method; 100 iterations, precision of 10^{-6}, 5% of tolerance and 1×10^{-4} convergence. The first-order deactivation rate constant obtained form linear regression of $\ln(A_r)$ *vs* t was used as initia values of the non-linear regression k_1 parameter. The non-linear regression parameters were constricted to positive numbers.

The reuse half-life time ($t_{1/2}$) of the biocatalyst, i.e., the operation time needed to reduce its original activity (non-deactivated enzyme) to 50% could then be determined according to the models fitted to the deactivation profiles. The reuse half-life time is determined trough interpolation or even trough extrapolation.

3. Results and discussion

One of the problems to be overcome on naringin bioconversion with naringinase is the low solubility of naringin in aqueous media (3 mM, 45 °C) which limits the productivity and yield of the enzymatic system. A straightforward solution to this problem is the application of biphasic or co-solvent systems.

3.1. Biphasic system

Towards the use of liquid–liquid biphasic conditions for naringin biocatalysis, a selection of different solvents was carried out according to their log P values and functionality. The following solvents were tested with the goal of increase naringin solubility: octane and n-hexane were representative of unbranched alkanes; cyclohexane of cyclic alkanes; 1-octanol, 2-octanol and 1-hexanol of alcohols; toluene and anisole of aromatic compounds; ethylic ether of ethers; ethyl acetate of esters; carbon tetrachloride and chloroform of halogenated solvents. Unsatisfactory results were afforded due to the low solubility of naringin in these non-polar solvents. Then, the biphasic design system was excluded for the bioprocessing of naringin, but these solvents will be useful in our future work of deglycosilation of more hydrophobic glycosides.

3.2. Aqueous co-solvent systems

Another approach to enhance naringin concentration in the enzymatic reaction media was the use of water co-solvation systems according to solvents relative permitivity. The main goal was to maximize naringin solubilization in the aqueous system, allowing naringinase with a high retention activity and stability.

In Table 1 are presented the different solvents tested, the respective dielectric constant and naringin solubility. From these results (Table 1) no solvent structure–naringin solubility relationship could be inferred. In fact, naringin showed a low solubility (<12.5 mM) in the following aqueous co-solvent systems: acetonitrile, 3-methyl-1-butanol, 2-methyl-2-butanol. Propylene carbonate, 1 and 2-propanol, 1and 2-butanol slightly improved naringin solubilization. Acetone, 1,2-dimetoxiethane, dimethyl sulfoxide, *N*,*N*-dimethylmethanamide, *N*,*N*-dimethyylcetamide, methanol, ethanol, tetrahydrofurane, 1,2-dimetoxiethane and 1,4-dioxane showed to be the best co-solvents, allowing a naringin solubilization superior to 100 mM.

Afterwards, the effect of these solvents (5%) were tested over the activity of α-L-rhamnosidase and β-D-glucosidase expressed by naringinase, using the hydrolysis reaction of the substrates *p*-nitrophenyl α-L-rhamnopyranoside and *p*-nitrophenyl β-D-glucopyranoside, respectively, at pH 4.0, 30 °C.

Table 1
Naringin solubility in different co-solvents systems at 25 °C.

Solvent	Dielectric constant	[Naringin] (mM)
Water	80.10	<12.5
Propylene carbonate	65.00	[12.5–25]
Dimethyl sulfoxide	47.20	[100–200]
N,*N*-Dimethylmethanamide	40.10	>200
N,*N*-Dimethylacetamide	38.80	>200
Methanol	36.60	>200
Acetonitrile	36.60	<12.5
Ethanol	25.30	[100–200]
Acetone	21.01	>200
1-Propanol	20.80	[25–50]
2-Propanol	20.18	[25–50]
1-Butanol	17.84	[12.5–25]
2-Butanol	17.26	[12.5–25]
3-methyl-1-butanol	15.63	<12.5
2-Butoxyethanol	9.57	[50–100]
Tetrahydrofurane	7.60	>200
1,2-Dimethoxyethane	7.40	>200
2-Methyl-2-butanol	5.97	<12.5
1,4-Dioxane	2.22	[100–200]

As it is shown in Table 2, α-L-rhamnosidase presented a greater (>50%) residual activity in the presence of 1,4-dioxane, 1,2-dimetoxiethane, tetrahydrofurane, acetone, ethanol, methanol and acetonitrile, while for β-D-glucosidase high activities (>50%) are attained with almost all the solvent tested, exception to 1-butanol, 3-methyl-1-butanol and 2-butoxyethanol. According to these preliminary studies, and considering the higher naringin solubility together with the higher residual activity of α-L-rhamnosidase and β-D-glucosidase expressed by soluble naringinase, eight solvents (dimethyl sulfoxide, *N*,*N*-dimethylmethanamide, methanol, ethanol, acetone, tetrahydrofurane, 1,2-dimethoxyethane and 1,4-dioxane) were chosen to be used in further stability and activity studies (Tables 3 and 4).

3.3. Solvent stability studies

Naringinase was immobilized on sol–gel matrices of tetramethoxysilane and glycerol, with a loading of 90% and less than 5% of enzyme leaching. In previous work these matrices were

Table 2
Residual activity of α-rhamnosidase and β-glucosidase expressed by soluble naringinase (625 mg L^{-1}), with 5% (v/v) of co-solvent, in acetate buffer (20 mM) pH 4.0, 30 °C.

Co-solvent	Residual activity	
	β-Glucosidase	α-Rhamnosidase
Propylene carbonate	0.34 ± 0.04	0.57 ± 0.03
Dimethyl sulfoxide	0.85 ± 0.06	1.01 ± 0.01
N,*N*-Dimethylmethanamide	0.52 ± 0.04	0.78 ± 0.05
N,*N*-Dimethylacetamide	0.54 ± 0.01	0.80 ± 0.04
Methanol	0.80 ± 0.06	0.92 ± 0.07
Acetonitrile	0.73 ± 0.04	0.90 ± 0.01
Ethanol	0.73 ± 0.01	0.83 ± 0.04
Acetone	0.64 ± 0.01	0.81 ± 0.02
1-Propanol	0.50 ± 0.02	0.52 ± 0.06
2-Propanol	0.72 ± 0.03	0.83 ± 0.08
1-Butanol	0.10 ± 0.01	0.40 ± 0.04
2-Butanol	0.48 ± 0.02	0.77 ± 0.04
3-Methyl-1-butanol	0.41 ± 0.01	0.08 ± 0.03
2-Butoxyethanol	0.26 ± 0.02	0.26 ± 0.02
Tetrahydrofurane	0.70 ± 0.02	0.65 ± 0.02
1,2-Dimethoxyethane	0.80 ± 0.04	0.81 ± 0.06
2-Methyl-2-butanol	0.46 ± 0.04	0.93 ± 0.06
1,4-Dioxane	0.58 ± 0.01	0.83 ± 0.06

(Average ± standard error, $n = 3$).

Table 3
Deactivation parameters of α-L-rhamnosidase expressed by soluble and immobilized naringinase in co-solvent systems.

Co-solvent [% (v/v)]	α-L-Rhamnosidase								$t_{1/2\ imm} \cdot t_{1/2\ sol}^{-1}$
	(Expressed by soluble naringinase)			(Expressed by sol–gel immobilized naringinase)					
	k_1 (h^{-1})	$t_{1/2\ sol}$	r^2	a_1	k_1 (h^{-1})	k_2 (h^{-1})	$t_{1/2\ imm}$	r^2	
Dimethyl sulfoxide									
0	0.19 ± 0.01	3.63 ± 0.31	0.96	1.29 ± 0.02	2.18 ± 0.29	0.21 ± 0.05	5.22 ± 0.57	0.96	1
2.5	0.32 ± 0.02	2.20 ± 0.16	0.91	1.58 ± 0.01	0.72 ± 0.13	0.20 ± 0.01	7.30 ± 0.26	0.95	3
5	0.32 ± 0.0	2.30 ± 0.05	0.94	1.39 ± 0.21	0.82 ± 0.26	0.12 ± 0.05	6.67 ± 0.99	0.99	3
10	0.33 ± 0.02	2.10 ± 0.12	0.87	1.17 ± 0.05	0.21 ± 0.09	0.13 ± 0.01	6.33 ± 0.12	0.94	3
N,*N*-Dimethylacetamide									
2.5	0.40 ± 0.02	1.75 ± 0.07	0.90	1.26 ± 0.39	1.87 ± 0.10	0.13 ± 0.01	7.97 ± 0.58	0.93	5
5	0.52 ± 0.01	1.33 ± 0.04	0.94	1.11 ± 0.05	4.00 ± 0.34	0.12 ± 0.10	5.00 ± 1.15	0.96	4
10	0.82 ± 0.02	0.84 ± 0.02	0.98	–	0.16 ± 0.02	–	4.23 ± 0.50	0.88	5
Methanol									
2.5	0.43 ± 0.01	1.60 ± 0.02	0.94	1.54 ± 0.22	5.00 ± 0.33	0.14 ± 0.03	8.53 ± 1.54	0.94	5
5	0.47 ± 0.02	1.47 ± 0.08	0.93	1.41 ± 0.13	2.00 ± 0.37	0.18 ± 0.06	5.77 ± 0.35	0.91	4
10	0.66 ± 0.02	1.05 ± 0.03	0.98	1.43 ± 0.09	3.23 ± 0.34	0.17 ± 0.01	6.57 ± 0.23	0.99	6
Ethanol									
2.5	0.37 ± 0.02	1.87 ± 0.10	0.88	1.67 ± 0.09	1.68 ± 0.38	0.20 ± 0.05	6.77 ± 0.95	0.96	4
5	0.53 ± 0.01	1.31 ± 0.04	0.87	1.48 ± 0.18	2.50 ± 0.16	0.23 ± 0.04	4.67 ± 0.37	0.97	4
10	1.16 ± 0.01	0.60 ± 0.03	0.96	–	0.13 ± 0.01	–	5.00 ± 0.57	0.94	8
Acethone									
2.5	0.45 ± 0.01	1.55 ± 0.03	0.94	1.42 ± 0.19	2.90 ± 0.74	0.19 ± 0.05	5.83 ± 1.53	0.95	4
5	0.47 ± 0.02	1.47 ± 0.06	0.93	1.22 ± 0.12	3.20 ± 0.44	0.17 ± 0.06	5.37 ± 0.77	0.95	4
10	0.86 ± 0.02	0.81 ± 0.02	0.96	1.27 ± 0.02	4.50 ± 0.42	0.17 ± 0.04	6.03 ± 1.13	0.95	7
Tetrahydrofuran									
2.5	1.02 ± 0.02	0.68 ± 0.01	0.99	1.47 ± 0.34	3.50 ± 0.36	0.20 ± 0.02	5.00 ± 0.70	0.97	7
5	1.57 ± 0.02	0.44 ± 0.01	0.99	1.13 ± 0.06	11.80 ± 0.12	0.15 ± 0.01	5.53 ± 0.22	0.95	13
10	5.33 ± 0.02	0.13 ± 0.01	0.99	–	0.25 ± 0.01	–	2.77 ± 0.12	0.93	21
1,2-Dimethoxyethane									
2.5	0.82 ± 0.02	0.85 ± 0.02	0.99	1.17 ± 0.08	3.70 ± 0.79	0.23 ± 0.08	4.03 ± 0.79	0.96	5
5	0.89 ± 0.02	0.78 ± 0.02	0.99	–	0.17 ± 0.01	–	4.20 ± 0.15	0.93	5
10	1.21 ± 0.02	0.57 ± 0.01	0.99	–	0.24 ± 0.02	–	2.87 ± 0.18	0.97	5
1,4-Dioxane									
2.5	0.31 ± 0.02	2.20 ± 0.06	0.85	1.38 ± 0.09	2.90 ± 0.40	0.19 ± 0.02	5.27 ± 0.35	0.98	2
5	0.48 ± 0.02	1.43 ± 0.07	0.94	1.16 ± 0.07	4.00 ± 0.12	0.20 ± 0.02	4.30 ± 0.10	0.98	3
10	1.00 ± 0.02	0.69 ± 0.05	0.99	–	0.24 ± 0.01	–	2.97 ± 0.18	0.86	4

Average ± standard error, $n = 3$.

re-used in 50 successive batches with no detectable loss of activity.

In this work, stability studies with α-L-rhamnosidase and β-D-glucosidase expressed by soluble and sol–gel immobilized naringinase were performed in aqueous co-solvent systems, at 0%, 2.5%, 5% and 10% (v/v), of dimethyl sulfoxide, acetone, *N*,*N*-dimethylacetamide, methanol, 1,2-dimetoxiethane, ethanol, tetrahydrofurane, 1,4-dioxane, using the procedure described in Section 2.7. In these aqueous co-solvent systems, the use of naringinase immobilized onto sol–gel matrices allowed higher residual activities of α-L-rhamnosidase and β-D-glucosidase with respect to the corresponding soluble enzyme. At the lowest co-solvent concentration, the immobilized biocatalyst was always more stable than the soluble one (Figs. 2 and 3). In the case of β-D-glucosidase a residual activity greater than 100% was attained with all co-solvents, even at higher concentrations (10%), with the exception to dimethyl sulfoxide (Fig. 3). A 1.5 increase in β-D-glucosidase residual activity was observed with the co-solvent 1,2-dimetoxiethane.

These results showed the protective effect of sol–gel matrices above the co-solvents, in the three concentrations tested, being this effect more pronounced for β-D-glucosidase (Fig. 3) than for α-L-rhamnosidase (Fig. 2), when compared with soluble enzyme.

The experimental plots of residual activity of soluble enzyme versus time were adjusted to exponential decays ($A_r = e^{-k_1 t}$), allowing the determination of k_1 and the half-life of the biocatalysts.

There was a good fit between experimental deactivation kinetics of α-L-rhamnosidase and β-D-glucosidase from the soluble naringinase and a simple exponential decay model was adjusted, allowing the calculation of k_1 and the half-life (Tables 5 and 6). In all the co-solvent systems tested an increase in the deactivation parameters k_1 with a corresponding decrease in half-lives were observed for soluble enzyme (Tables 5 and 6).

An activity increase was seen whenever the sol–gel immobilized naringinase was re-used for the first time. This fact may be explained by some glycerol release from the sol–gel matrix used during the immobilization procedure. This glycerol release may lead to a better substrate access to the immobilized enzyme, increasing enzyme activity after the first run. The deactivation of α-L-rhamnosidase and β-D-glucosidase from the immobilized naringinase was well described by the series-type enzyme deactivation scheme (1) involving first-order steps in a deactivation sequence as well as an active intermediate.

The deactivation curves for the immobilized enzyme were fitted to the two-step deactivation model proposed by Henley and Sadana [17]:

$$E \xrightarrow{k_1} E_1 \xrightarrow{k_2} E_2$$

The kinetics of enzyme (E) deactivation 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 and second deactivation rate coefficients, respectively; $\alpha_1 = [E]\{([E_1]/[E]) \times$

Table 4
Deactivation parameters of β-glucosidase expressed by soluble and immobilized naringinase in co-solvent systems.

Co-solvent [% (v/v)]	β-Glucosidase										$t_{1/2\ imm} \cdot t_{1/2\ sol}^{-1}$
	(Expressed by soluble naringinase)					(Expressed by sol–gel immobilized naringinase)					
	α_1	k_1 (h^{-1})	k_2 (h^{-1})	$t_{1/2\ sol}$	r^2	α_1	k_1 (h^{-1})	k_2 (h^{-1})	$t_{1/2\ imm}$	r^2	
Dimethyl sulfoxide											
0	0.76 ± 0.03	2.84 ± 0.84	0.074 ± 0.01	6.18 ± 0.55	0.99	1.29 ± 0.01	2.18 ± 0.79	0.21 ± 0.00	33.6 ± 4.0	0.99	5
2.5	–	0.19 ± 0.01	–	3.72 ± 0.12	0.89	1.15 ± 0.06	26.27 ± 8.21	0.037 ± 0.02	20.7 ± 3.7	0.91	6
5	–	0.20 ± 0.01	–	3.10 ± 0.14	0.95	1.15 ± 0.03	3.16 ± 0.10	0.053 ± 0.01	13.8 ± 1.0	0.91	4
10	–	0.32 ± 0.00	–	2.16 ± 0.03	0.95	1.16 ± 0.10	0.65 ± 0.08	0.071 ± 0.02	12.9 ± 0.5	0.88	6
N,*N*-Dimethylacetamide											
2.5	–	0.25 ± 0.01	–	2.72 ± 0.08	0.89	1.34 ± 0.24	24.92 ± 7.48	0.039 ± 0.01	25.4 ± 2.6	0.88	9
5	–	0.42 ± 0.01	–	1.84 ± 0.05	0.93	1.30 ± 0.05	2.70 ± 0.78	0.056 ± 0.01	15.3 ± 1.4	0.97	8
10	–	1.40 ± 0.09	–	0.49 ± 0.03	0.96	1.43 ± 0.43	0.93 ± 0.08	0.104 ± 0.08	11.7 ± 1.6	0.99	24
Methanol											
2.5	–	0.27 ± 0.01	–	2.54 ± 0.06	0.90	1.43 ± 0.17	30.84 ± 9.53	0.059 ± 0.01	17.4 ± 1.5	0.92	7
5	–	0.30 ± 0.02	–	2.33 ± 0.16	0.92	1.38 ± 0.02	3.37 ± 0.18	0.056 ± 0.00	18.0 ± 2.1	0.84	8
10	–	0.44 ± 0.03	–	1.56 ± 0.09	0.85	1.11 ± 0.10	1.77 ± 0.53	0.047 ± 0.01	17.5 ± 2.2	0.99	11
Ethanol											
2.5	–	0.28 ± 0.01	–	2.47 ± 0.06	0.89	1.40 ± 0.13	23.15 ± 2.31	0.063 ± 0.01	17.3 ± 2.1	0.87	7
5	–	0.31 ± 0.01	–	2.25 ± 0.11	0.86	1.37 ± 0.03	30.00 ± 9.71	0.050 ± 0.01	18.3 ± 1.5	0.87	8
10	–	0.94 ± 0.01	–	0.74 ± 0.02	0.94	1.27 ± 0.07	3.43 ± 0.67	0.051 ± 0.00	18.0 ± 1.0	0.97	24
Acetone											
2.5	–	0.32 ± 0.02	–	2.14 ± 0.07	0.92	1.27 ± 0.06	26.54 ± 8.54	0.027 ± 0.00	31.7 ± 2.2	0.93	15
5	–	0.38 ± 0.01	–	1.80 ± 0.06	0.93	1.35 ± 0.10	0.87 ± 0.05	0.058 ± 0.02	20.9 ± 4.5	0.92	12
10	–	0.95 ± 0.03	–	0.73 ± 0.03	0.98	1.61 ± 0.08	0.18 ± 0.43	0.148 ± 0.01	13.4 ± 2.4	0.80	18
Tetrahydrofuran											
2.5	–	0.30 ± 0.01	–	2.31 ± 0.10	0.95	1.34 ± 0.01	20.82 ± 0.77	0.046 ± 0.01	24.7 ± 5.0	0.96	11
5	–	0.41 ± 0.02	–	1.69 ± 0.10	0.83	1.45 ± 0.02	2.07 ± 0.74	0.069 ± 0.00	16.1 ± 1.2	0.97	10
10	–	2.12 ± 0.04	–	0.33 ± 0.01	0.97	1.29 ± 0.02	3.43 ± 0.17	0.050 ± 0.00	19.2 ± 1.0	0.90	59
1,2-Dimethoxyethane											
2.5	–	0.25 ± 0.00	–	2.77 ± 0.04	0.92	1.56 ± 0.02	2.58 ± 0.97	0.063 ± 0.00	17.8 ± 0.9	0.98	6
5	–	0.33 ± 0.00	–	2.06 ± 0.02	0.98	1.46 ± 0.10	4.51 ± 0.77	0.066 ± 0.01	16.5 ± 2.3	0.97	8
10	–	0.53 ± 0.04	–	1.30 ± 0.08	0.92	1.36 ± 0.04	5.16 ± 0.96	0.052 ± 0.01	17.7 ± 2.9	0.96	14
1,4-Dioxane											
2.5	–	0.21 ± 0.02	–	3.36 ± 0.33	0.92	1.37 ± 0.03	3.46 ± 0.74	0.047 ± 0.00	21.3 ± 2.2	0.99	6
5	–	0.28 ± 0.00	–	2.50 ± 0.03	0.85	1.20 ± 0.02	30.12 ± 0.82	0.043 ± 0.00	20.2 ± 0.5	0.91	8
10	–	0.47 ± 0.01	–	1.46 ± 0.04	0.94	1.19 ± 0.02	20.17 ± 0.11	0.084 ± 0.01	19.2 ± 3.7	0.89	13

Average ± standard error, $n = 3$.

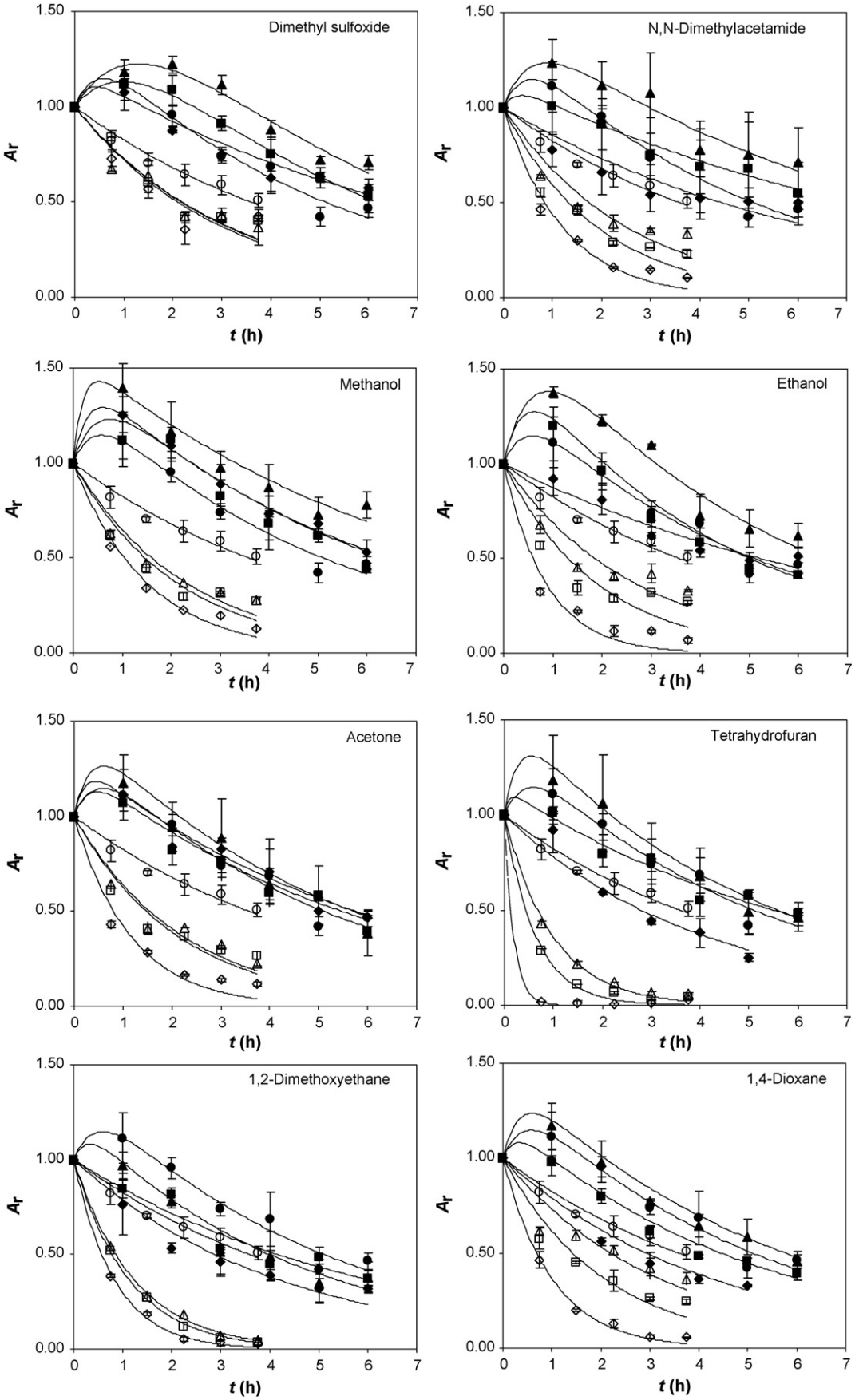

Fig. 2. Stability of α-L-rhamnosidase expressed by soluble and immobilized naringinase in co-solvent systems for the hydrolysis of *p*-nitrophenyl α-L-rhamnopyranoside. 0%, 2.5%, 5%, 10% (v/v) co-solvent percentage respectively, with soluble (○ △ □ ◊) and immobilized (● ▲ ■ ♦) naringinase.

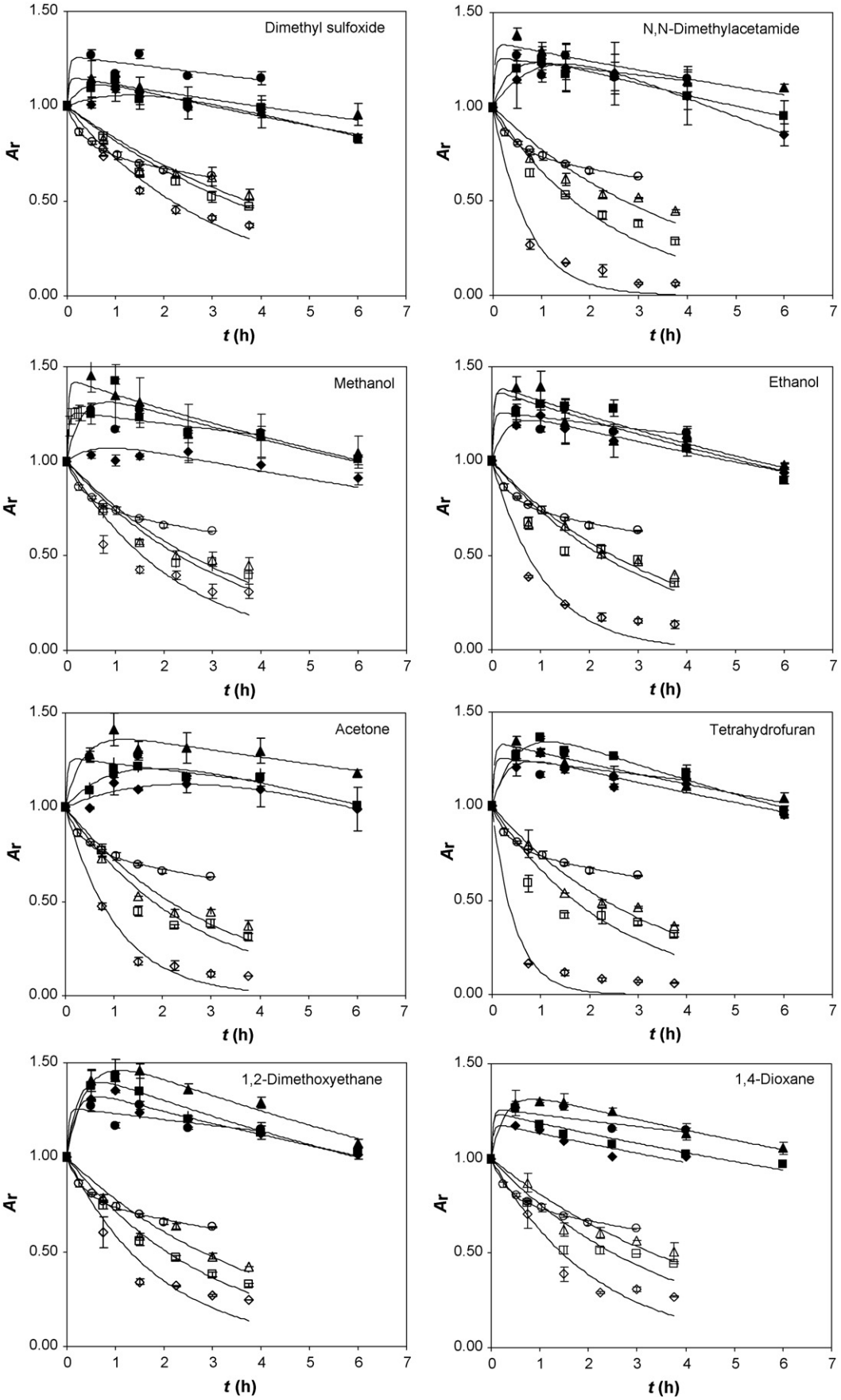

Fig. 3. Stability of β-D-glucosidase expressed by soluble and immobilized naringinase in co-solvent systems for the hydrolysis of *p*-nitrophenyl β-D-glucopyranoside. 0%, 2.5%, 5%, 10% (v/v) co-solvent percentage respectively, with soluble (○ △ □ ◊) and immobilized (● ▲ ■ ♦) naringinase.

Table 5
Kinetic parameters for naringin hydrolysis with soluble naringinase, in 10% aqueous co-solvent systems in acetate buffer pH 4.0, 45 °C.

Co-solvent	k_{cat} (μmol min^{-1} mg^{-1})	K_M (mM)	$k_{cat}.K_M^{-1}$ (L min^{-1} g^{-1})	r^2
Water	2.05 ± 0.14	1.92 ± 0.27	1.07	0.99
1,2-Dimetoxiethane	2.52 ± 0.09	4.26 ± 0.30	0.59	0.99
1,4-Dioxane	1.91 ± 0.17	5.15 ± 0.22	0.37	0.99
Tetrahidrofurane	1.46 ± 0.18	8.00 ± 2.72	0.18	0.97

Average ± standard error, $n = 3$.

Table 6
Kinetic parameters for naringin hydrolysis with soluble naringinase, in 1,2-dimetoxiethane, in different concentrations, acetate buffer pH 4.0, 45 °C.

1,2-Dimetoxiethane (%)	k_{cat} (μmol min^{-1} mg^{-1})	K_M (mM)	$k_{cat}.K_M^{-1}$ (L min^{-1} g^{-1})	r^2
0	2.40 ± 0.18	2.21 ± 0.33	1.08	0.99
1	2.45 ± 0.12	3.03 ± 0.42	0.81	0.98
2	2.54 ± 0.10	3.58 ± 0.53	0.71	0.98
3	2.60 ± 0.08	4.41 ± 0.59	0.59	0.98

Average ± standard error, $n = 3$.

100} and $\alpha_2 = [E]\{([E_2]/[E]) \times 100\}$. Henley and Sadana [17] had derived a weighted-average activity (A_r) expression for a nonzero specific activity for the final state E_2:

$$A_r = \alpha_2 + \left[1 + \frac{\alpha_1 k_1}{k_2 - k_1} - \frac{\alpha_2 k_2}{k_2 - k_1}\right] e^{-k_1 t} - \left[\frac{\alpha_1 k_1}{k_2 - k_1} - \frac{\alpha_2 k_1}{k_2 - k_1}\right] e^{-k_2 t} \quad (1)$$

The parameters k_1, k_2, α_1, α_2 and the half-life of the biocatalysts were calculated (Tables 3 and 4). We considered, improved enzyme stability as a decrease in the deactivation parameters k_1 or k_2 with a corresponding increase in half-life or as an increase in enzyme residual activity, α_1.

The improved stabilization of α-L-rhamnosidase expressed by sol–gel immobilized in co-solvents is shown by the increase in half-lives from 0.10 to 8.5 h (Table 3). It can be highlight in the use of tetrahydrofurane in concentrations of 2.5%, 5.0% and 10.0%, which originated an increase of 7-, 13- and 21-fold, on half-lives, respectively, with respect to the corresponding soluble enzyme. This is an example of the high protective effect of the sol–gel (TMOS + glycerol) to the enzyme naringinase over different co-solvent concentrations.

In the case of β-D-glucosidase activity, expressed by sol–gel immobilized naringinase, an increase in the half-life almost 100-fold was observed, respectively from 0.33 (soluble enzyme) to 31.7 h (Table 4).

For all the aqueous co-solvents systems an increase greater than 4 in half-lives with immobilized enzyme was observed (Tables 3 and 4). To the higher co-solvent concentration used (10%) the sol–gel protective effect was much more pronounced, as shown by the increase in half-lives, e.g. 11-fold with ethanol, 13-fold with 1,4-dioxane, 14-fold with 1,2-dimetoxiethane, 18-fold to acetone, 24-fold with *N*,*N*-dimethylacetamide and the best one 59-fold with tetrahydrofuran.

For all co-solvent systems tested, we found very stable states, E_1, with high residual activities (α_1), over 111%, a decrease in the deactivation parameters k_2 with the consequent increase in half-lives for the sol–gel immobilized naringinase (Tables 3 and 4). These results support an improved stabilization assumption.

The immobilization of naringinase through sol–gel technique improved both α-L-rhamnosidase and β-D-glucosidase resistance against deactivation caused by the co-solvent presence. Enhanced biocatalyst stabilization, in all solvent range tested, was achieved through immobilization. The different behaviors, in terms of stability in certain solvent conditions, using soluble and immobilized enzyme, may arise from the different mechanisms of inactivation, when solvent conditions are changed. Glycerol is known as a stabilizer of the protein structure of enzymes and in this case, of naringinase immobilization onto sol–gel (TMOS + glycerol), may prevent unfolding at high concentrations of organic co-solvents.

Brena [18] showed that with higher co-solvent concentrations, the immobilized *Escherichia coli* β-galactosidase onto glutaraldehyde–agarose, 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, corresponding to decrease in half-life of the immobilized derivative with respect to the soluble form. In the other cases, such as 18% (v/v) dioxane, the half-life was increased from 8 for the soluble enzyme to 100 h for the derivative and in 36 (%) dimethylformamide, 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 was dependent on the properties of the co-solvent [18].

Relatively higher activities of α-glucosidase from *S. cerevisiae* were reported in the presence of co-solvents with low log *P* values such as methanol and dimethyl sulfoxide [19], namely in 30% dimethyl sulfoxide and in 30% methanol, where respectively 25% and 12.5% of the activity in pure water was detected. α-Glucosidase was also more stable in the presence of dimethyl sulfoxide than methanol, respectively 100% and 20% of the activity remained. These authors [19] stabilized α-glucosidase by immobilization onto macroporous poly(GMA-co-EGDMA). The immobilized enzyme had 4 and 10-fold higher half-lives than the soluble one in dimethyl sulfoxide and methanol, respectively [19]. In other work [20], water-miscible co-solvents, like dimethyl sulfoxide, were used to increase substrate solubility [20], however, the catalytic activity of P450 was significantly lower. The effect of methanol, ethanol, dimethyl sulfoxide, and acetonitrile on the catalytic activities of nine human cDNA-expressed cytochrome P-450s in aqueous co-solvent systems was tested [21].

3.4. Kinetic studies

Kinetic studies of naringin hydrolysis were carried out in free solvent and 10% aqueous co-solvent systems of 1,2-dimetoxietane, 1,4-dioxane and tetrahidrofurane (pH = 4.0, 45 °C).

A maximum naringin concentration of 2.5 mM was solubilised in the free solvent aqueous system, while increased naringin concentrations were used in the 10% aqueous co-solvent systems, with a maximum concentration, of 5 mM in 1,2-dimetoxietane and 15 mM in 1,4-dioxane and tetrahidrofurane, respectively (Fig. 4). The less toxic co-solvents were respectively,

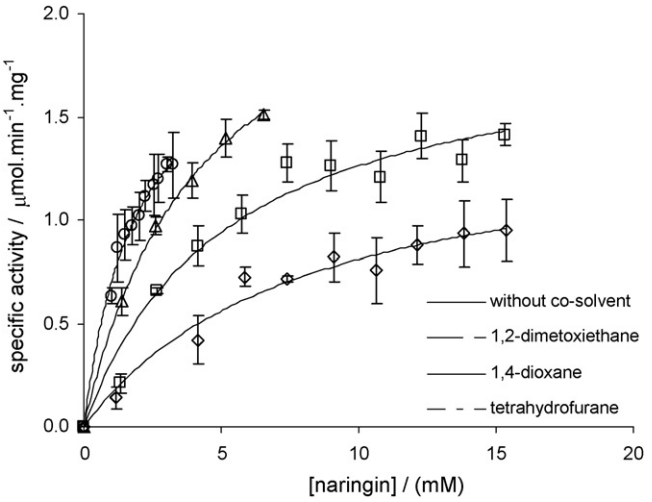

Fig. 4. Michaelis–Menten kinetics of naringin hydrolysis by naringinase, in different aqueous co-solvent systems (10%) (acetate buffer 20.0 mM, pH = 4.0, 45 °C, [naringinase] = 250 mg L^{-1}).

1,2-dimetoxiethane, 1,4-dioxane, and tetrahydrofurane. In fact, the enzymatic reaction extension increased 6-, 8- and 10-fold in the co-solvents, respectively, with tetrahydrofurane, 1,4-dioxane and 1,2-dimetoxiethane.

Kinetic constants were evaluated on naringin hydrolysis by naringinase in these different aqueous co-solvent systems. 1,4-Dioxane and 1,2-dimetoxiethane showed a higher naringinase specific activity, being considered the most favourable (Fig. 4). Naringin presented a higher solubility in 1,4-dioxane (Table 1), but a higher k_{cat} and a lower K_M was observed with 1,2-dimetoxiethane (Table 5).

Different concentrations of 1,2-dimetoxiethane (1%, 2% and 3%) were tested using different naringin concentrations, allowing kinetic constant evaluation (Fig. 5). An improvement in enzymatic reaction progression was attained with the increased 1,2-dimetoxiethane concentrations, in reaction medium, respectively, 6-fold for 1%, 8-fold for 2% and 10-fold for 3%, when compared to solvent free aqueous medium. According to the results presented in Fig. 5 and Table 6, high concentration of 1,2-dimetoxiethane in aqueous systems led to a slight increase in k_{cat} and higher K_M. The k_{cat} was much less affected with the presence of the co-solvent, than the apparent K_M, also a sharp decrease of the catalytic efficiency (k_{cat}/K_M) was observed.

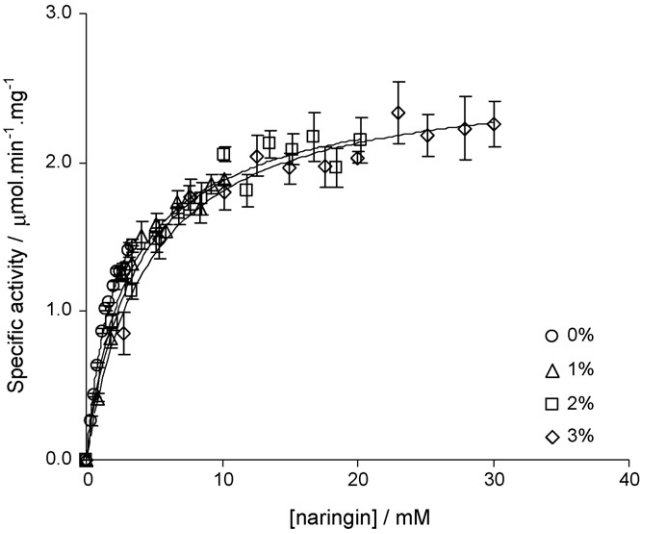

Fig. 5. Michaelis–Menten kinetics of naringin hydrolysis by naringinase, with different concentrations of 1,2-dimetoxiethane (1%, 2% and 3%) (acetate buffer 0.02 M, pH = 4, 45 °C, [naringinase]≈250 mg L^{-1}).

An increase in Michaelis–Menten constant, K_M for the hydrolysis of naringin in the aqueous co-solvent system of 1,2-dimetoxiethane in water reflects changes in the affinity of the enzyme for the substrate, which is common in reactions where hydrophobic interactions are involved in the enzyme-substrate complex formation. Similar results were obtained in other works, respectively, increased Michaelis–Menten constant for the hydrolytic activity of trypsin [22] and of α-chymotrypsin [23] in co-solvent systems. However, in the co-solvent system with 1,2-dimetoxiethane (3%) a 10-fold increase in naringinase enzymatic reaction extension was observed.

4. Conclusions

Aqueous co-solvent systems were developed to increase naringin solubility, with a high enzyme retention activity. In all the co-solvent systems tested a decrease in the deactivation parameters k_1 and k_2 with a corresponding increase in half-lives were observed for sol–gel immobilized naringinase, reflecting an improved stabilization.

For all the aqueous co-solvents systems an increase greater than 4 in half-lives with immobilized enzyme was observed. To the higher co-solvent concentration used (10%) the sol–gel protective effect was much more pronounced, as shown by the increase in half-lives of β-D-glucosidase expressed by naringinase, respectively, 13-, 14-, 24- and 59-fold 1,4-dioxane, 1,2-dimetoxiethane, *N*,*N*-dimethylacetamide and tetrahydrofuran.

In the co-solvent system with 1,2-dimetoxiethane (3%) a 10-fold increase in naringinase enzymatic reaction extension was observed.

In conclusion, with the sol–gel naringinase immobilization we achieved enzyme improved stabilization in the presence of co-solvent in all range of concentrations tested. This enzyme stabilization may be due to glycerol, known to be a stabilizer of the protein structure and in the case of naringinase immobilization onto sol–gel (TMOS + glycerol) may prevent unfolding at high concentrations of organic co-solvents. These are high innovative and sounding results showing the protective effect of immobilization onto sol–gel (TMOS + glycerol) matrices, which is a great advantage on non-conventional biocatalysis.

Acknowledgements

Helder Vila Real was supported by Fundação para a Ciência e Tecnologia, Portugal. Grant no. SFRH/BD/30716/2006.

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