Process Biochemistry 46 (2011) 2330–2335

Contents lists available at SciVerse ScienceDirect

Process Biochemistry

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

Operational stabilization of fungal α-rhamnosyl-β-glucosidase by immobilization on chitosan composites

Lucrecia Piñuel, Laura S. Mazzaferro, Javier D. Breccia*

INCITAP-CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas), Departamento de Química, Facultad de Ciencias Exactas y Naturales, Universidad Nacional de La Pampa (UNLPam), Av. Uruguay 151, (6300) Santa Rosa, La Pampa, Argentina

ARTICLE INFO

Article history:
Received 26 July 2011
Received in revised form
15 September 2011
Accepted 19 September 2011
Available online 28 September 2011

Keywords:
Dimethylsulfoxide
Glycoside hydrolase
Rutinose
Hesperidin
Hesperidin methylchalcone
Polyethyleneimine
Metal catalyzed oxidation

ABSTRACT

The diglycosidase α-rhamnosyl-β-glucosidase from *Acremonium* sp. DSM24697 was immobilized by adsorption and cross-linking. The supports screened included beads of chitosan composites (gelatin, arabic gum, silica gel), epoxy-activated agarose and chitosan, and macroporous polyvinyl-alcohol cryogel. The chitosan–silica gel beads were selected because of the highest immobilization efficiency obtained and their morphological properties (diameter 1.67 ± 0.99 mm, circularity 0.81 ± 0.05). The optimization of the immobilization—coating with polyethyleneimine, changes in the enzyme load—improved the immobilization efficiency up to 18%. The practical use of the enzyme deals with low water solubility substrates. The higher K_M for the immobilized enzyme—8 mM vs. 1.8 mM hesperidin for the free enzyme—indicated that substrate diffusion limits the enzymatic reaction. The solvent dimethylsulfoxide (50%, v/v) was added in order to increase the substrate solubility, and 80% activity was retained (1 h, 60 °C) in contrast with the complete inactivation of the free form. The stability of the immobilized catalyst was extended to metal catalyzed oxidation where the enzyme was fully preserved in harsh conditions such as 1 mM $CuSO_4$ at 60 °C during 1 h.

1. Introduction

Glycoside hydrolases (EC 3.2.1.-) are a widespread group of enzymes, which hydrolyze the glycosidic bond, and from a biotechnological point of view, they find extended applications in bulk biotransformations as well as in fine chemistry for modification of biologically active compounds [1]. We recently reported the enzyme α-rhamnosyl-β-glucosidase (EC 3.2.1.168), a distinctive glycosidase because of its substrate specificity for 7-*O*-rutinosides as well as its mode of action as a diglycosidase. It cleaves the flavonoid hesperidin and other 7-*O*-rutinosylated flavonoids in the heterosidic bond to yield the disaccharide rutinose (α-rhamnopyranosyl-β-glucopyranose) and the corresponding aglycone [2]. On account of its hydrolytic activity, α-rhamnosyl-β-glucosidase has potential use for industrial processing of plant-based foods [3]. On the other hand, the ability of the enzyme to transglycosylate rutinosyl units to OH-acceptor compounds, using as starting material an abundant and inexpensive by product of citric industry, such as hesperidin and hesperidin methylchalcone, makes this enzyme a promissory system for the development of new drugs [25].

Stable glycosidases with reutilization capacity is a key requirement for economically relevant biotransformations [4]. The low stability of the proteins in water-soluble organic co-solvents constitutes a problem for using them in synthetic reactions [5]. Many applications demand the use of these solvents in the reaction mixtures to increase the kinetic constant and yield of the processes by raising substrates solubility. In that way, several glycosidases have been immobilized onto a number of supports in view of their application in food processing and fine chemistry [6–8]. Moreover, the immobilization of the biocatalyst allows the kinetic control of a given process avoiding the final thermodynamic equilibrium that could lead with the hydrolysis of the synthesized products. In this work we have assessed different strategies to obtain an immobilized preparation of α-rhamnosyl-β-glucosidase stable in operational conditions. The polymer chitosan was chosen as the main constituent of the support based on its compatibility with proteins, chemical stability and the non-toxic character [9].

2. Materials and methods

2.1. Chemicals

Hesperidin, hesperidin methylchalcone, rutinose, chitosan, glutaraldehyde 50% (v/v), polyethyleneimine PEI (MW 60 kDa), dimethylsulfoxide (DMSO), were purchased from Sigma Chemical (St. Louis). All other chemicals were from standard sources.

* Corresponding author. Tel.: +54 2954 436787x26; fax: +54 2954 432535.
E-mail address: javierbreccia@exactas.unlpam.edu.ar (J.D. Breccia).

1359-5113/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.procbio.2011.09.014

2.2. Production and partial purification of α-rhamnosyl-β-glucosidase

The α-rhamnosyl-β-glucosidase was purified from the culture supernatant of *Acremonium* sp. DSM24697 as described before [2]. The procedure includes salt precipitation and hydrophobic interaction chromatography (butyl-agarose) to yield 30.4% of the initial activity and purified 45.7 fold. The product was desalted and freeze-dried before storing at −18 °C.

2.3. Enzyme assay

For quantification of α-rhamnosyl-β-glucosidase activity, each reaction contained 450 μl of substrate 0.11% (w/v) hesperidin in 50 mM sodium citrate buffer pH 5.0 and 50 μl of suitably diluted enzyme solution. The reaction was performed for 1 h at 60 °C and stopped by adding 500 μl of 3,5-dinitrosalicylic acid (DNS) [10]. For immobilized enzyme activity, each reaction contained 50 mg of beads in 500 μl substrate. The immobilized enzyme was removed before adding DNS. The tubes were placed in a boiling water bath for 10 min and cooled before measuring the absorbance at 540 nm. One unit of α-rhamnosyl-β-glucosidase activity was defined as the amount of enzyme required to release 1 μmol of rutinose/min. The kinetic parameters for the immobilized enzyme were determined using hesperidin and hesperidin methylchalcone as substrates. The initial velocities of the reaction were plotted against substrate concentration and the data were adjusted using the Michaelis–Menten equation by the least squares method.

2.4. Preparation of chitosan composites beads

Chitosan flakes (0.5 g) were dissolved in 1 M acetic acid (50 ml). The additive 50% (w/w) (silica gel, arabic gum and gelatin) was added to the chitosan solution. It yielded a viscous solution that was dropped at a constant rate, into a neutralizing solution containing 15% (w/v) NaOH and 96% ethanol in a volume ratio of 4:1 with magnetic stirring at 300 rpm. The formed beads were washed with deionized water until neutral pH was reached, and stored at 4 °C for further uses. The 32-bit color images of the beads were split into red, green and blue (RGB) components using the software ImageJ (National Institutes of Health, USA: http://rsb.info.nih.gov/ij/). Images corresponding to the green component were chosen for morphological characterization due to the highest signal to noise ratio. The morphological characterization of the beads was performed with the free program ImageJ 1.42q (National Institutes of Health). Size distribution was fitted with the Gauss curve:

$$y = y_0 + \frac{A}{w\sqrt{\pi/2}} e^{-2(x-x_c)^2/w^2}$$

where y_0 is the medium diameter of the beads and w represents the size dispersion. Circularity was calculated by the following equation:

$$\text{Circularity} = 4\pi \frac{\text{area}}{\text{perimeter}^2}$$

A circularity value of 1.0 indicates a perfect circle. As the value approaches 0.0, it indicates an increasingly elongated polygon.

2.5. Preparation of PEI–chitosan–silica gel beads

Polyethylenimine (PEI) (MW 60 kDa) was adsorbed to the support throughout electrostatic interactions with the silica (SiO_2) fraction [11]. PEI-coated beads were prepared as described Pessela et al. with slight modifications [12]. Wet support (2.2 g) was suspended in 135 ml of 10% (w/v) PEI. The suspension was maintained under mild stirring at 25 °C for 3 h. Then, the beads were treated with 10 mg/ml sodium borohydride (2 h at 25 °C). Finally, the suspension was filtered and washed consecutively with 100 mM sodium acetate buffer pH 5.0, 100 mM sodium borate buffer pH 7.8, and an excess of distilled water. The beads were stored at 4 °C in an aqueous solution containing 50% (v/v) ethanol. The presence of the PEI-coating onto the beads was confirmed by incubating them in 1 mM Cu_2SO_4 solution, visualizing the blue complex formed with copper ions.

2.6. Optimization of the immobilization of α-rhamnosyl-β-glucosidase

The α-rhamnosyl-β-glucosidase from *Acremonium* sp. DSM24697 was immobilized by adsorption-cross linking using freeze-dried pure enzyme or culture broth. The enzyme (0.017–0.067 U/ml) was incubated (18 h at room temperature) with 400 mg of wet chitosan composites beads suspended in 3.4 ml of 50 mM sodium phosphate buffer pH 7.0. The cross-linking was carried out incubating the mixture with 0.5% (v/v) glutaraldehyde (GA) at room temperature for 30 min [6]. Afterwards, the beads were washed thoroughly with distilled water.

The immobilization yield (YI) and the immobilization efficiency (EI) were defined as:

$$\text{YI} = \frac{A_{\text{loaded}} - A_{\text{unbound}}}{A_{\text{loaded}}} \times 100$$

$$\text{EI} = \frac{A_{\text{beads}}}{A_{\text{loaded}} - A_{\text{unbound}}} \times 100$$

where A_{loaded} is the enzyme activity loaded, A_{unbound} is the enzyme activity remaining in the supernatant, and the activity bound to the support (A_{beads}) was calculated as the units of enzyme activity per gram of wet beads

2.7. Stability in the presence of a co-solvent (dimethylsulfoxide) and Cu^{2+} ions

The immobilized enzyme (25 mg beads) was incubated with 0–50% (v/v) of DMSO or 1 mM $CuSO_4$ at 60 °C during 1 h in 50 mM citrate buffer (pH 5.0). Then, the support was washed exhaustively with distilled water to remove rests of DMSO or Cu^{2+} ions. Determination of the residual activity was carried out as described above. The sample without additives was fixed as 100% activity.

3. Results and discussion

3.1. Immobilization of Acremonium sp. DSM24697 α-rhamnosyl-β-glucosidase on chitosan and chitosan composites beads

The enzyme α-rhamnosyl-β-glucosidase was immobilized onto chitosan beads by adsorption and cross-linking. The covalent binding of adsorbed α-rhamnosyl-β-glucosidase with the cross-linking agent (glutaraldehyde) was essential to avoid the enzyme leakage, although it was deleterious for the expressed activity on the support (A_{beads}) (data not shown), as it had been demonstrated for the immobilization of other glycoside hydrolases [6,8,13].

Purification process steps of proteins are preferably avoided for immobilization purposes. In this study, both the supernatant of the submerged culture and the purified enzyme of *Acremonium* sp. DSM24697 were used as biocatalyst source for the evaluation of the process. The immobilization efficiency (EI) on chitosan beads was low, with EI of 0.04 and 0.5% for the supernatant and the pure enzyme, respectively (Fig. 1). Moreover, the chitosan beads are not suitable for some operational conditions, i.e. agitated reactor, because of the low mechanical stability in presence of shear forces. In order to improve the immobilization efficiency and the mechanical stability, the beads were blended with several additives that were chosen in basis of their different chemical nature: silica gel, gelatin and arabic gum. The immobilization showed a better performance for chitosan-composites beads than for chitosan beads (Fig. 1). The efficiency was increased 3–5 fold for pure protein and 25–75 fold for culture supernatant (Fig. 1). In this case, the higher efficiency for the supernatant samples suggested that some components of the supernatant might have a protective effect on the structure of α-rhamnosyl-β-glucosidase. The binding of a protein in presence of other compounds such as brown colored compounds and polysaccharides may impair the immobilization yield [14]. By the other side, the co-immobilization of two or more proteins has been previously demonstrated to lower the immobilization stress by competing for the binding sites of the support, promoting a non-distorting conformation of the immobilized enzyme [15,16].

The chitosan-composites beads were morphologically characterized as is shown in Table 1. The chitosan–silica beads showed the highest circularity and homogeneity—as evidenced by size dispersion around the media. Moreover, chitosan–silica gel beads were previously reported as more resistant material in comparison with the other chitosan-composites used, which is in agreement with its inorganic nature [13]. Because of the higher immobilization efficiency obtained and their physical properties we selected chitosan–silica gel beads for further studies.

Table 1
Morphological characterization of the chitosan composite beads.

Additive	Diameter (mm)	Circularity
Arabic gum	2.24 ± 1.03	0.74 ± 0.09
Gelatin	3.25 ± 1.20	0.60 ± 0.09
Silica-gel	1.67 ± 0.99	0.81 ± 0.05

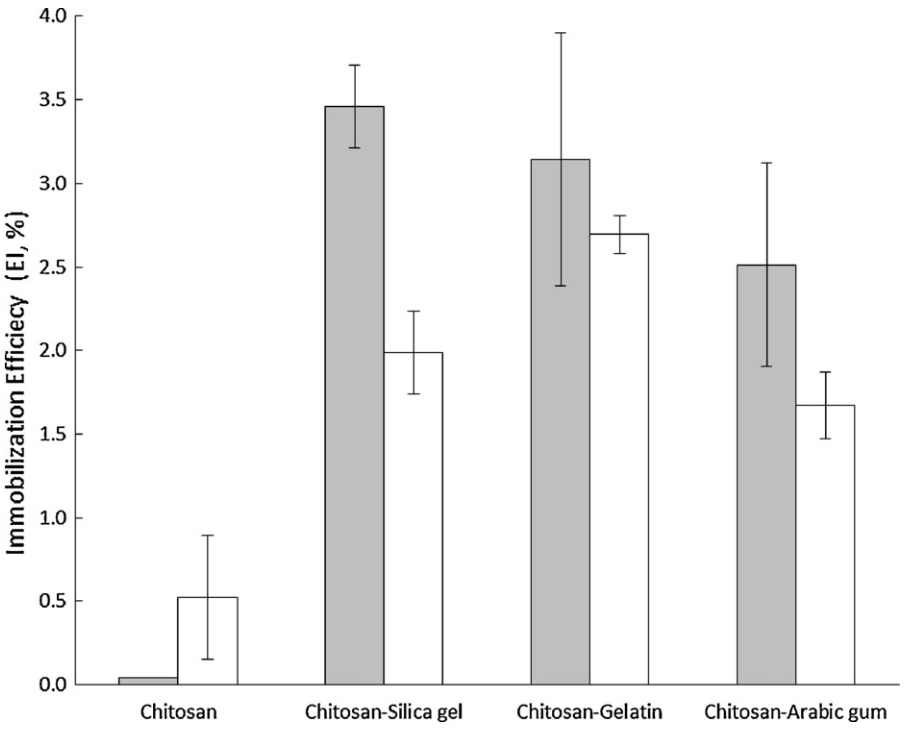

Fig. 1. Immobilization of α-rhamnosyl-β-glucosidase on chitosan composite beads using (■) culture supernatant and (□) pure α-rhamnosyl-β-glucosidase of *Acremonium* sp. DSM24697.

3.2. Chitosan–silica beads coated with polyethyleneimine

The recycling of the biocatalyst was evaluated during five uses of 1 h-length at 60 °C (Fig. 2). After the first use, the enzymatic activity on the support, A_{beads}, was diminished to 15% of the initial activity. Interestingly, the A_{beads} was not modified in the subsequent cycles. In addition, during long-term storage, the immobilized enzyme was shown to retain 100% of the activity after 65 days at 8 °C.

In order to prevent the decrease of the enzyme activity during the first cycle, the beads were coated with polyethyleneimine.

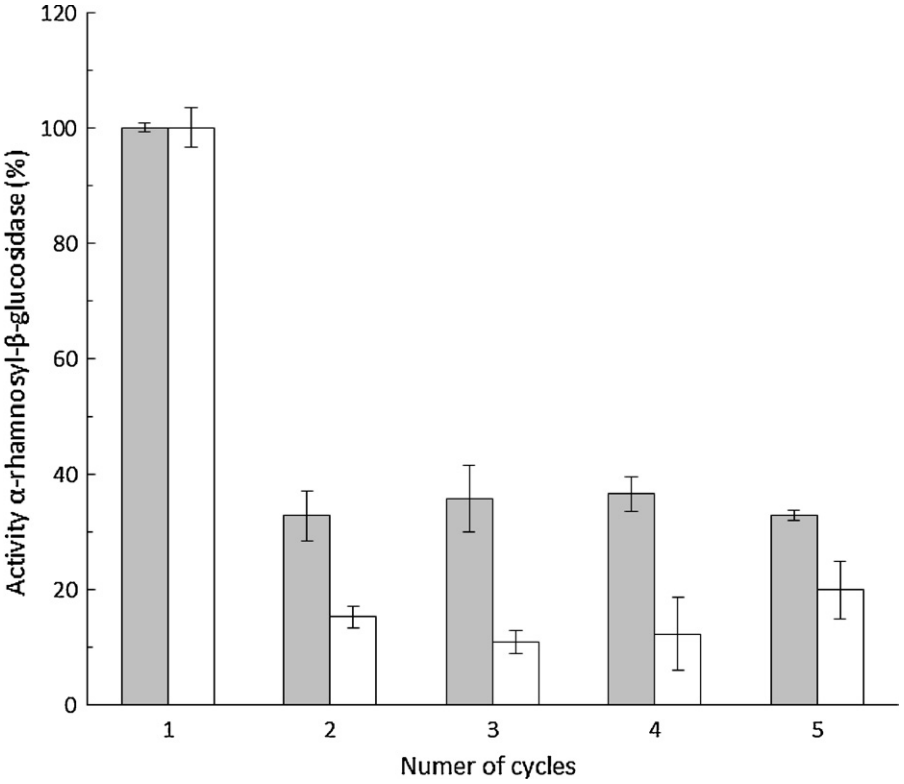

Fig. 2. Operational stability of the immobilized α-rhamnosyl-β-glucosidase from *Acremonium* sp. DSM24697 using crude extract as enzyme source and beads of (■) chitosan–silica–PEI and (□) chitosan–silica. Hundred percent correspond to the activity on the support before the recycling: 0.17 U/g for chitosan–silica–PEI and 0.13 U/g for chitosan–silica beads.

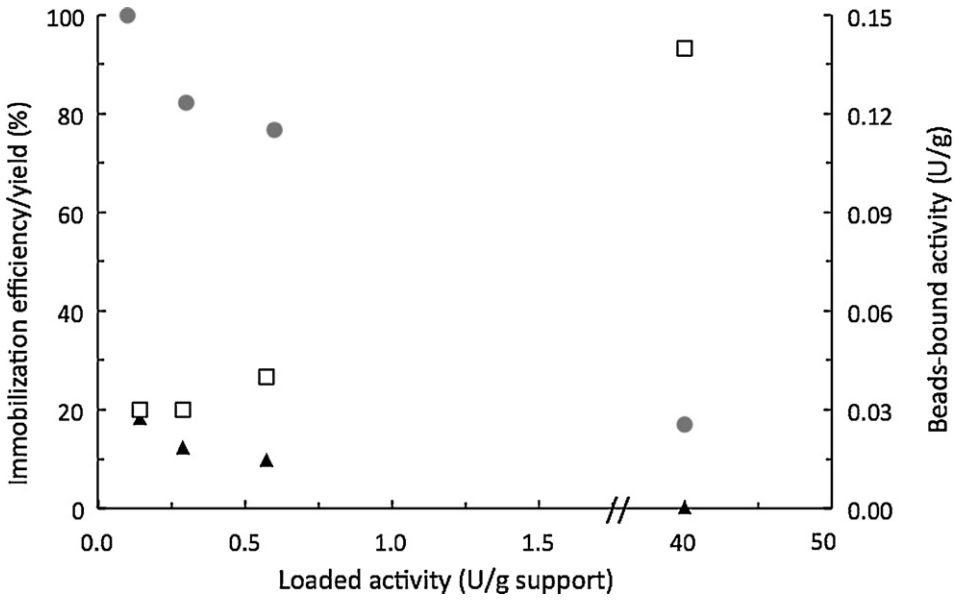

Fig. 3. Influence of enzyme load on the inmobilization of purified α-rhamnosyl-β-glucosidase from *Acremonium* sp. DSM24697. (□) Beads bound activity (U/g), (▲) Immobilization efficiency (%) and (●) Immobilization yield (%).

Table 2
Effect of temperature on the leakage of α-rhamnosyl-β-glucosidase immobilized on chitosan–silica–PEI beads.

Temperature (°C)	Enzyme leakage (U/g)
30	ND
40	0.63 ± 0.005
50	1.44 ± 0.095
60	1.16 ± 0.080

The activity was measured in the supernatant after incubating the beads at different temperatures for 1 h.

This polymer gets adsorbed on silica dispersions producing flocculated slurry in a wide range of pH values [11]. In this case it produced a stable layer on the chitosan–silica beads most likely by ionic interaction with the silica fraction. PEI is a polycationic polymer ($pK_a \sim 9$) that forms complexes with acidic proteins due to electrostatic forces [17,18]. Since the isoelectric point of α-rhamnosyl-β-glucosidase is acidic (pI 5.7), it is expected to interact with PEI in the conditions of the immobilization procedure (pH 7).

The adsorption of enzymes onto composites with flexible polymers containing a very high density of ion exchange moieties would promote a very strong adsorption on a flexible coating bed [7]. Remarkably, the residual activity after the first use was increased from 15% to 35% for the PEI-coated support (Fig. 2). The 65% loss of activity during the first cycle was attributed to a temperature-dependent leakage found between 40 and 60 °C but not below 30 °C (Table 2). Although the immobilization efficiency was not modified in the presence of PEI, the importance of the PEI coating resides in the maintenance of the enzyme bound to the support at high temperatures. Previously, Pessela et al. [7] reported that the adsorption strength was much higher using PEI supports, where desorption of a β-galactosidase could be prevented even at high ionic strength (0.5 M NaCl at pH 7).

The binding capacity of chitosan–silica–PEI beads was assessed loading different amounts of partially purified α-rhamnosyl-β-glucosidase. The A_{beads} increased as the amount of loaded enzyme was augmented, but the immobilization efficiency was diminished (Fig. 3). The immobilization yield (YI) was found to be 100% when 0.1 U/g were loaded; and the immobilization efficiency was 18%, e.g. 0.03 U/g support. This behavior brought about a tradeoff regarding the optimization of the conditions for the immobilization process. The obtainment of 5 times higher activity on the support required ~400 times higher enzyme loading, and the immobilization efficiency was as low as 2%. The profile of the immobilization efficiency observed as the enzyme loading was augmented agrees with the results previously reported for glycoside hydrolases by several authors [19,20]. The immobilization efficiency was low in comparison with a β-glucosidase from *Issatchenkia terricola* immobilized in Eupergit C250L [19]. Eupergit C250L is a commercial support composed of epoxy-activated polyacrylate-based beads. The replacement of the support by beads of macroporous polyvinyl-alcohol cryogel and epoxy-activated supports (agarose and chitosan beads) did not improve the efficiency of α-rhamnosyl-β-glucosidase immobilization in comparison with chitosan–silica–PEI beads (data not shown).

3.3. Kinetic parameters of immobilized α-rhamnosyl-β-glucosidase

The enzyme α-rhamnosyl-β-glucosidase was shown to be active against flavonoid 7-*O*-rutinosides, with the highest affinity for hesperidin (K_M 1.7 mM), a compound scarcely soluble in water (0.324 mM) [2]. The diffusion hindrance of the substrate is one of the phenomena that would explain the low activity expressed on chitosan–silica–PEI beads. The kinetic parameters of the immobilized enzyme were determined using hesperidin and hesperidin

Table 3
Influence of immobilization process on kinetic parameters.

Substrate		Free [2]	Immobilized	$K_M(i)/K_M(f)$
Hesperidin (H)	K_M	1.8	8.0	4.4
Hesperidin methylchalcone (HMC)	K_M	8.7	38.7	4.4
	V_{max}(HMC)/V_{max}(H)	2	13	

(*i*) Immobilized; (*f*) free enzyme.

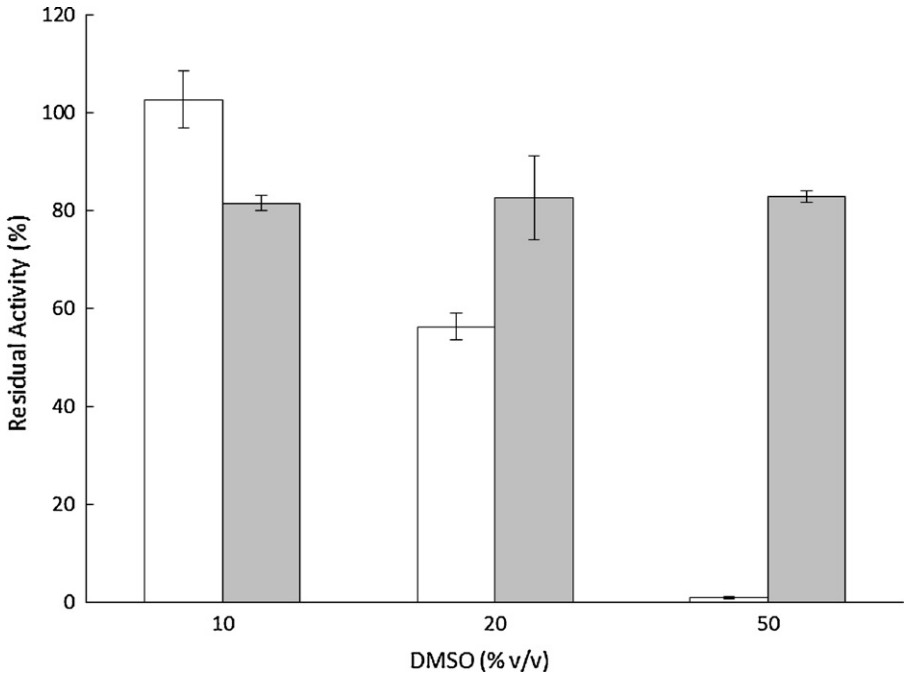

Fig. 4. Stability of α-rhamnosyl-β-glucosidase in presence of dimethylsulfoxide at high temperature. The biocatalyst was incubated for 1 h at 60 °C with different concentration of DMSO. (□) Free α-rhamnosyl-β-glucosidase, (■) immobilized α-rhamnosyl-β-glucosidase. Hundred percent corresponds to the activity without additives 0.89 U/ml for free enzyme and 0.08 U/g for the immobilized catalyst.

methylchalcone—a flavonoid 7-*O*-rutinoside that is water-soluble (>300 mM) (Table 3). The apparent Michaelis constants for the immobilized catalyst were found to be 4.4 times higher for both substrates, in comparison with the free enzyme. The change in the affinity of the enzyme for its substrate is probably caused by structural changes in the enzyme introduced by the immobilization procedure and by the lower accessibility of the substrate to the active site of the immobilized enzyme [20]. By the other hand, the V_{max} for hesperidin was significantly reduced in comparison with hesperidin methylchalcone for the immobilized catalyst. This result indicated that the diffusion rate of the substrate limits the enzymatic reaction. As a consequence, the diffusion limitation is greater for the less soluble substrate.

3.4. Immobilization promotes protein stability against metal-catalyzed oxidation

Metal catalyzed oxidation (MCO) is associated to protein modifications promoting aggregation, irreversible amino acid modifications, increased susceptibility to proteolysis and/or fragmentation of the protein backbone [17,21]. As a consequence, traces of metals are involved in activity losses during storage conditions. α-Rhamnosyl-β-glucosidase was incubated for 1 h at 60 °C in presence of 1 mM $CuSO_4$. The soluble form of the enzyme losts 97% of the initial activity while no activity losses were detected for the immobilized enzyme, regardless the coating with PEI (Table 4). PEI is a chelating polymer for several metal ions like iron and copper and it was reported as a stabilizer against MCO for soluble proteins [17,18,21]. Interestingly, both the polymer and the enzyme are located in a restricted layer around the beads and the former promotes a higher concentration of the metal at the surroundings of the enzyme environment without affecting the activity. These results indicate that immobilization process promotes stabilization of α-rhamnosyl-β-glucosidase against MCO in harsh conditions like 60 °C and 1 mM $CuSO_4$.

Table 4
Effect of metal catalyzed oxidation on the activity of α-rhamnosyl-β-glucosidase.

State	Residual activity (%)
Free enzyme	2.9 ± 0.3
Immobilized coated support	105.1 ± 8.5
Immobilized uncoated support	92.4 ± 4.0

The residual activity was measured after incubating the biocatalyst with 1 mM $CuSO_4$ at 60 °C for 1 h.

3.5. Organic co-solvent in the reaction mixture increases substrate solubility and affects enzyme stability

The practical use of the immobilized α-rhamnosyl-β-glucosidase deals with the low water solubility of its main substrate, hesperidin. The inclusion of an organic co-solvent in the reaction medium has been frequently used to increase the synthetic efficiency of glycosidase-catalyzed processes; the two most commonly employed organic co-solvents in this respect being dimethylsulfoxide (DMSO) and acetone. However, enzymes are usually unstable under such conditions [22]. The stability of immobilized α-rhamnosyl-β-glucosidase in presence of the aprotic solvent DMSO is particularly interesting. The transglycosylation as well as hydrolysis reactions are favored in presence of this solvent, due to the increment in the solubility of the substrate [23]. The residual activity was studied after 1 h incubation at 60 °C in presence of DMSO (Fig. 4). The residual activity of the free-enzyme was strongly dependent on the DMSO concentration. A reduction of 40% of the initial activity was found after incubation in 20% (v/v) DMSO and the activity was practically undetectable after incubation with 50% (v/v) DMSO. Previous reports had been demonstrated that immobilization of a protein on a support with a polymeric bed (dextran) would change the enzyme from a hydrophobic environment to a hydrophilic one promoting the enzyme stability in presence of organic solvents [24]. In this case, the immobilized enzyme maintained 82% of the activity independently of DMSO concentration after the exposure at high temperature.

4. Concluding remarks

The enzyme α-rhamnosyl-β-glucosidase was reported recently by our group for the hydrolysis of the flavonoid hesperidin into the aglycone hesperetin and the disaccharide rutinose [2]. The prone oxidation character of the enzyme and its low stability in presence of organic solvent would limit the use of the enzyme. The immobilization process onto chitosan–silica–PEI beads showed a good performance to overcome the mentioned drawbacks for biotransformation reactions.

Acknowledgements

This work was supported by Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Universidad Nacional de La Pampa (UNLPam), and Agencia Nacional de Promoción Científica y Técnica (ANPCyT) of Argentina.

References

[1] Monti D, Candido A, Cruz Silva MM, Křen V, Riva S, Danieli B. Biocatalyzed generation of molecular diversity: selective modification of the saponin asiaticoside. Adv Synth Catal 2005;347:1168–74.
[2] Mazzaferro L, Piñuel L, Minig M, Breccia JD. Extracellular monoenzyme deglycosylation system of 7-*O*-linked flavonoid β-rutinosides and its disaccharide transglycosylation activity from *Stilbella fimetaria*. Arch Microbiol 2010;192:383–93 [Erratum, Arch Microbiol 2011;193:461].
[3] Genovés S, Gil JV, Vallés S, Casas JA, Manzanares P. Assessment of the aromatic potential of palomino fino grape must using glycosidases. Am J Enol Vitic 2005;56:188–91.
[4] Gavrilescu M, Chisti Y. Biotechnology—a sustainable alternative for chemical industry. Biotechnol Adv 2005;23:471–99.
[5] Orrillo AG, Ledesma P, Delgado OD, Spagna G, Breccia JD. Cold-active α-l-rhamnosidase from psychrotolerant bacteria isolated from a sub-Antarctic ecosystem. Enzyme Microb Technol 2007;40:236–41.
[6] Martino A, Pifferi PG, Spagna G. Immobilization of β-glucosidase from a commercial preparation. Part 2. Optimization of the immobilization process on chitosan. Process Biochem 1995;31:287–93.
[7] Pessela B, Fernández-Lafuente R, Fuentes M, Vián A, García JL, Carrascosaa A, Mateo C, Guisán JM. Reversible immobilization of a thermophilic β-galactosidase via ionic adsorption on PEI-coated Sepabeads. Enzyme Microb Technol 2003;32:369–74.
[8] Spagna G, Andreani F, Salatelli I, Romagnoli D, Pifferi PG. Immobilization of α-L-arabinofuranosidase on chitin and chitosan. Process Biochem 1998;33:57–62.
[9] Krajewska B. Application of chitin- and chitosan-based materials for enzyme immobilizations: a review. Enzyme Microb Technol 2004;35:126–39.
[10] Miller GL. Use of dinitrosalisylic acid (DNS) for determination of reducing sugars. Anal Chem 1959;31:426–8.
[11] Lavanya A, Yee-Kwong L. Interactions of PEI (polyethylenimine)–silica particles with citric acid in dispersions. Colloid Polym Sci 2011;289:237–45.
[12] Pessela B, Betancor L, López-Gallego F, Torres R, Dellamora-Ortiz GM, Alonso-Morales N, Fuentes M, Fernández-Lafuenta R, Guisán JM, Mateo C. Increasing the binding strength of proteins to PEI coated supports by immobilizing at high ionic strength. Enzyme Microb Technol 2005;37:295–9.
[13] Spagna G, Barbagallo R, Greco E, Manenti I, Pifferi PG. A mixture of purified glycosidases from *Aspergillus niger* for oenological application immobilized by inclusion in chitosan gels. Enzyme Microb Technol 2002;30:80–9.
[14] Martino A, Durante M, Pifferi PG, Spagna G, Bianchi G. Immobilization of β-glucosidase from a commercial preparation. Part 1. A comparative study of natural supports. Process Biochem 1995;31:281–5.
[15] Morana A, Mangione A, Maurelli L, Fiume I, Paris O, Cannio R, Rossi M. Immobilization and characterization of a thermostable β-xylosidase to generate a reusable biocatalyst. Enzyme Microb Technol 2006;39:1205–13.
[16] Minakshi, Pundir CS. Co-immobilization of lipase, glycerol kinase, glycerol-3-phophate oxidase and peroxidase onto aryl amine glass beads affixed on plastic strip for determination of triglycerides in serum. Indian J Biochem Biophys 2008;45:111–5.
[17] Andersson MM, Breccia JD, Hatti-Kaul R. Stabilizing effect of chemical additives against oxidation of lactate dehydrogenase. Biotechnol Appl Biochem 2000;32:145–53.
[18] Mazzaferro LS, Breccia JD, Andersson MM, Hitzmann B, Hatti-Kaul R. Polyethyleneimine–protein interactions and implications on protein stability. Int J Biol Macromol 2010;47:15–20.
[19] González-Pomboa P, Fariña L, Carraub F, Batista-Viera F, Brena BM. A novel extracellular β-glucosidase from *Issatchenkia terricola*: isolation, immobilization and application for aroma enhancement of white Muscat wine. Process Biochem 2011;46:385–9.
[20] Hernaiz MJ, Crout DHG. Immobilization/stabilization on Eupergit C of the β-galactosidase from *B. circulans* and an α-galactosidase from *Aspergillus oryzae*. Enzyme Microb Technol 2000;27:26–32.
[21] Breccia JD, Andersson M, Hatti-Kaul R. The role of poly(ethyleneimine) in stabilization against metal-catalized oxidation of proteins: a case study with lactate dehydrogenase. Biochim Biophys Acta 2002;1570:165–73.
[22] Heidecke CD, Parsons TB, Fairbanks AJ. Endohexosaminidase-catalysed glycosylation with oxazoline donors: effects of organic co-solvent and pH on reactions catalysed by Endo A and Endo M. Carbohydr Res 2009;344:2433–8.
[23] Mazzaferro LS, Piñuel L, Breccia JD. Synthesis of 4-methylumbelliferyl-rutinoside: a new fluorogenic substrate for diglycosidases, submitted for publication.
[24] Irazoqui G, Giacomini C, Batista-Viera F, Brena B. Hydrophilization of immobilized model enzyme suggests a widely applicable method for enhancing proteins stability in polar organic co-solvents. J Mol Catal B: Enzym 2007;46:43–51.
[25] Mazzaferro LS, Breccia JD. Functional and biotechnological insights into diglycosidases. Biocatal Biotrans 2011;29:103–12.