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Production of Hesperetin Using a Covalently Multipoint Immobilized Diglycosidase from *Acremonium* sp. DSM24697

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Key Words

Biotransformation · Biocatalysis · Hesperidin · Flavonoids · Citrus by-products

Abstract

The diglycosidase α-rhamnosyl-β-glucosidase (EC 3.2.1.168) from the fungus *Acremonium* sp. DSM24697 was immobilized on several agarose-based supports. Covalent multipoint immobilization onto glyoxyl-activated agarose was selected as the more stable preparation at high concentration of dimethyl sulfoxide (DMSO) and high temperature. The optimal conditions for the immobilization process involved an incubation of the enzyme with agarose beads containing 220 μmol of glyoxyl groups per gram at pH 10 and 25°C for 24 h. The hydrolysis of hesperidin carried out in 10% v/v DMSO at 60°C for 2 h reached 64.6% substrate conversion and a specific productivity of 2.40 mmol h^{-1} g^{-1}. Under these conditions, the process was performed reutilizing the catalyst for up to 18 cycles, maintaining >80% of the initial activity and a constant productivity 2.96 ± 0.42 μmol^{-1} h^{-1} g^{-1}. To the best of our knowledge, such productivity is the highest achieved for hesperetin production through an enzymatic approach.

Flavone glycosides are one of the most abundant secondary metabolites in citrus species. These molecules impact on almost every citrus fruit's production and processing; for instance, they contribute to the bitter taste and also to juice clouding [Manthey and Grohmann, 1996]. Deglycosylation of different flavone glycosides is a challenge in food technology for debittering and clarifying fruit juices [Genovés et al., 2005; Hemingway et al., 1999; Wang et al., 2001]. On the other hand, several biological activities have been reported for the diverse backbones (2-phenyl-1,4-benzopyrone) of unglycosylated flavones [Benavente-García et al., 1997; Di Majo et al., 2005]. For instance, hesperetin, the aglycone of hesperidin, showed analgesic, anti-inflammatory and antioxidant properties, and it can also be used as synthon for the production of dyes and sweeteners [Di Majo et al., 2005].

Structurally, the flavonoid hesperidin is formed by rutinose (6-*O*-α-L-rhamnopyranosyl-β-D-glucopyranoside) linked to the aglycone hesperetin (fig. 1). There are few bioprocesses that efficiently hydrolyze hesperidin to yield hesperetin [Mandalari et al., 2006; Scaroni et al., 2002]. Most of them involve sequential steps catalyzed by at least two monoglycosidases – α-L-rhamnosidase (EC 3.2.1.40) and β-glucosidase (EC 3.2.1.21) – to remove the residues

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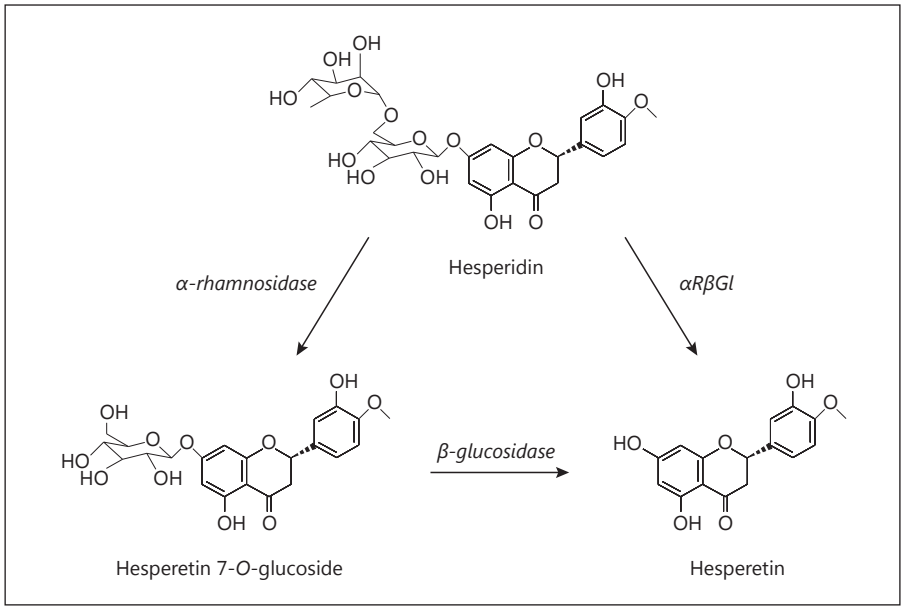

Fig.1. Pathways for enzymatic deglycosylation of hesperidin to yield hesperetin: via hesperetin-7-*O*-glucoside by two specific monoglycosidases, α-rhamnosidase (EC 3.2.1.40) and β-glucosidase (EC 3.2.1.21), and via one-step deglycosylation through αRβGl (EC 3.2.1.168).

of rhamnose and glucose, respectively (fig. 1). Recently, a novel diglycosidase from *Acremonium* sp. DSM24697 able to deglycosylate hesperidin in a single step, rendering rutinose and hesperetin as reaction products, was described. This enzyme, α-rhamnosyl-β-glucosidase (αRβGl; EC 3.2.1.168), is a distinctive endoglycosidase specific for 7-*O*-rutinosilate flavonoids (fig. 1) [Mazzaferro et al., 2010]. It also enables the transglycosylation of rutinosyl units using the flavonoids hesperidin or hesperidin methyl chalcone as rutinose donors, which are abundant and inexpensive by-products of the citrus industry [Mazzaferro et al., 2012]. The resulting rutinosyl derivatives have potential application in the pharmaceutical as well as the food industry [Mazzaferro and Breccia, 2011, 2012].

Biotransformations are optimized in order to simplify the processes and, depending on the characteristics of the biocatalyst, immobilization could be a convenient approach, especially for those enzymes that support harsh physicochemical conditions during immobilization and do not require cofactors for the catalysis [Ribeiro, 2011; Sheldon, 2007]. Covalent multipoint attachment on porous supports has been a suitable system for many enzymes, promoting protein stabilization [Mateo et al., 2005]. This work deals with the development of a stable preparation of αRβGl by covalent multipoint immobilization, focusing on the organic phase catalysis for hesperetin production.

Results and Discussion

Immobilization of αRβGl

The immobilization of αRβGl was designed to produce hesperetin in one single enzymatic process with the possibility of recycling the fungal biocatalyst as much as possible (fig. 1). To select an adequate immobilization procedure, the enzyme αRβGl was attached to agarose-based supports using four different ligands (table 1). It was quantitatively immobilized on the carrier containing nickel ions (Ag-Ni^{2+}), but was inactive on the support. Since this enzyme was reported as a prone oxidation protein, the inactivation on Ag-Ni^{2+} might be due to metal catalyzed oxidation or an improper position of the biocatalyst on the support [Piñuel et al., 2011]. In contrast, the reversi-ble immobilization on agarose activated with ethylenediamine (Ag-A) showed a lower yield (37.8%). When this preparation was covalently cross-linked to make the attachment irreversible, the yield improved to 65% and the immobilization efficiency dropped 55%, probably due to the known deleterious effect of glutaraldehyde (table 1) [Reshmi and Sugunan, 2012]. The same immobilization chemistry, ionic absorption followed by glutaraldehyde cross-linking, was inefficient on agarose coated with polyethylenimine (Ag-PEI). Ag-PEI immobilized >90% of the loaded protein; however, the activity exhibited on the support was vastly reduced (>80%) after cross-linking with glutaraldehyde. A comparable phenomenon was previously described when the enzyme was

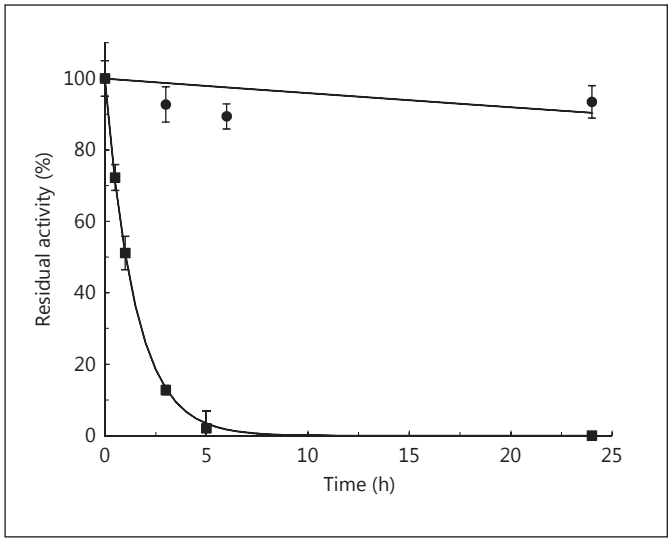

Fig. 2. The stability of soluble αRβGl incubated in 50% v/v DMSO at 25°C (●) and 50°C (■). The measurements of residual activity were performed after removing the solvent DMSO. The 100% activity corresponds to samples without DMSO containing 1.2 U/ml.

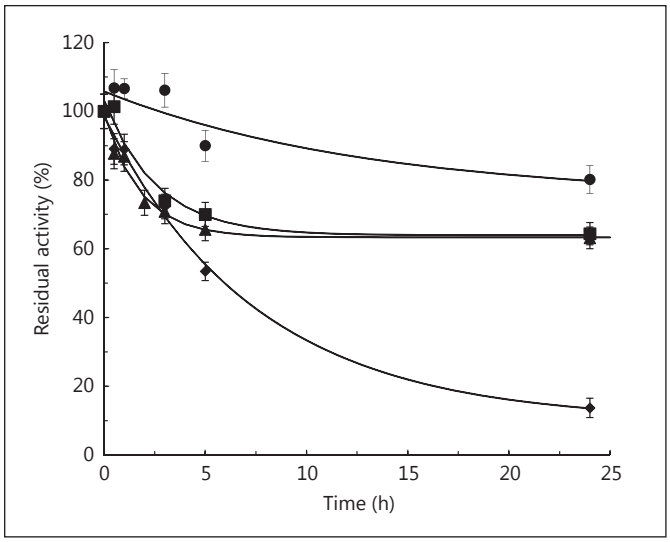

Fig. 3. The time course inactivation of different immobilized preparations of αRβGl in a medium containing 50% v/v DMSO at pH 5.0 and 40°C: soluble enzyme (▲), Ag-G (●), Ag-CB (◆) and Ag-A (■) cross-linked with glutaraldehyde. The 100% activity corresponds to samples without DMSO containing 0.83 U/ml for the soluble enzyme and 0.60, 0.79 and 0.39 U/g for the immobilized preparations, respectively.

immobilized on chitosan beads coated with PEI and cross-linked with glutaraldehyde [Piñuel et al., 2011]. On the other hand, unipunctual covalent attachment to cyanogen bromide agarose (Ag-CB) and multipunctual attachment to glyoxyl agarose (Ag-G) was performed through the N-terminal and lysine residues, respectively. The immobilization yield was significantly higher for Ag-CB. Nonetheless, both covalent derivatives exhibited similar immobilization efficiencies of around 64%, which surpassed the other procedures assayed as well as those previously reported (18% efficiency) for cross-linked PEI-chitosan beads of 1.67 ± 0.99 mm in diameter (table 1) [Piñuel et al., 2011].

Table 1. Immobilization of αRβGl on four different agarose derivatives

Support	YI, %	Immobilized activity, U/g	Expressed activity, U/g	EI, %
Ag-G	60.6	0.93	0.61	65.4
Ag-CB	90.3	1.25	0.79	62.9
Ag-Ni^{+2}	97.6	1.03	ND	0
Ag-A	37.8	0.40	0.13	32.8
Ag-A[a]	65.6	0.86	0.39	45.4
Ag-PEI[a]	93.0	1.47	0.16	11.1

All data are mean values of 3 separate experiments (error value <5%). YI and EI are defined in equations 1 and 2. ND = Not detected.

[a] Derivatives cross-linked with 0.5% v/v glutaraldehyde.

Stability of αRβGl Preparations

The polar aprotic solvent dimethyl sulfoxide (DMSO) is miscible in water and, at low concentration (<5% v/v), favored the catalysis of αRβGl by increasing the solubility of the substrate hesperidin [Mazzaferro et al., 2012]. Hence, the stability of the soluble enzyme was assessed by incubating it at high concentrations of the cosolvent (50%v/v DMSO) at two different temperatures and measuring the residual activity in the absence of DMSO (fig. 2). In this condition, the soluble enzyme was highly stable at low temperature (25°C) with an estimated half-life >160 h, but it was exponentially unfolded at 50°C ($t_{½}$ = 1 h). The destabilizing effect of protein structure by increment of the hydrophobic character of the medium at high DMSO concentration and temperature is a known phenomenon [Arakawa et al., 2007]. As a result, the stability of three αRβGl-insoluble preparations (Ag-CB, Ag-A + glutaraldehyde and Ag-G) was evaluated under 50% v/v aqueous solution of DMSO at 40°C. Figure 3

Table 2. Reaction media engineering for hesperetin production using αRβGl immobilized on Ag-G

Cosolvent, %	Reaction temperature, °C	Substrate, mM	Product, mM	Substrate conversion, %	Productivity[a], μmol h^{-1} g^{-1}
10	50	0.52	0.35	67.9	2.82±0.07
10	60	0.52	0.34	64.6	3.36±0.19
50	40	5	3.63	72.4	3.02±0.01
75	25	15	0	0	0
75	40	15	0	0	0
40:20[b]	40	10	1.19	11.9	0.99±0.04

[a] Productivity per gram of support; the preparation contained 1.5 mg of pure αRβGl per gram of solid carrier.
[b] Corresponding to 40% v/v DMSO and 20% v/v 2-butanone.

shows that Ag-G resulted in the more stable catalyst (estimated $t_{½}$ >70 h), the Ag-A preparation showed a similar profile to the soluble protein (estimated $t_{½}$ >25 h), while the Ag-CB ($t_{½}$ = 6 h) was the more unstable preparation. This implies that this enzyme, as well as several other enzymes reported, was highly stabilized by multipoint covalent immobilization with a half-life approximately three times higher in comparison with the soluble protein [Mateo et al., 2006]. Although knowledge of the primary and secondary structure of αRβGl is limited, the stabilization achieved by immobilization on Ag-G would suggest an important number of lysine residues fairly exposed in the protein surface and ready to react with the glyoxyl groups of the support [Mazzaferro et al., 2010].

The immobilization procedure onto Ag-G can be optimized by adjusting different variables, such as the time under alkaline conditions and the density of reactive groups on the support [Mateo et al., 2006]. The immobilization protocol was modified by changing the time of incubation at pH 10 and the stability of the resulting preparations was then evaluated in 50% v/v DMSO at 50°C. In all cases the immobilization yield was around 60%, but the stability of the preparation varied significantly. For 2 and 7 h of incubation at pH 10, the resulting preparations showed half-lives between 3 and 4 h, while the preparation incubated for 24 h doubled those values (data no shown). The longer time of incubation seems to endorse a larger number of covalent bounds and consequently an increment in the rigidity of the peptide, rendering higher stability in the presence of solvent and high temperature [Pedroche et al., 2007]. These results are in agreement with the lower stability exhibited by the Ag-CB derivative, where the peptide is attached unipunctually to the support (fig. 3) [Mateo et al., 2005]. Furthermore, the density of reactive groups on the support surface was assessed with Ag-G preparations containing 150 or 220 μmol of glyoxyl groups per gram. The half-life of the resulting derivatives incubated in 50% v/v DMSO at 50°C increased 4-fold for the support with a higher density of glyoxyl groups (data not shown). Therefore, the matrix with 220 μmol of glyoxyl groups per gram and the immobilization protocol with an incubation time at pH 10.0 of 24 h were applied in further experiments.

Engineering the Reaction Media to Improve Hesperetin Yield

The Ag-G αRβGl preparation was used to hydrolyze the 7-*O*-rutinosylated flavonoid, hesperidin, in several homogeneous systems (fig. 1; table 2). The biotransformation was carried out in the presence of different concentrations of DMSO to overcome the low solubility of hesperidin in water (0.32 mM) [Mazzaferro et al., 2010]. To increase the soluble substrate concentration, 50% v/v DMSO was used, dissolving up to 5 mM of hesperidin. Nevertheless, at a high cosolvent concentration, as well as high temperature, the environment becomes deleterious, even for the immobilized preparation of the catalyst. Therefore, the reaction was carried out at a lower temperature (40°C), obtaining 3.63 mM of hesperetin. Although this product concentration was 10-fold higher than that obtained in 10% v/v DMSO, the operational cycle at 50% v/v DMSO took 24 h to reach the plateau of maximum substrate conversion (72%), drastically reducing the productivity of the process (table 2). The conversion value (~70%) might be due to product inhibition issues, as has been reported elsewhere for other glycosidases [Ellenrieder et al., 1998]. In this way, other solvents (DMF, acetonitrile, ethyl acetate) were also evaluated, but hesperidin was not as soluble as in DMSO. For instance, the mixture of DMSO and 2-butanone dissolved up to 10 mM of hesperidin, but the substrate conversion was low (12%), probably due to a deleterious effect of the cosolvent mixture on the biocatalyst. Although higher concentrations of DMSO increase hesperidin solubility up to 15 mM, it was also detrimental to the hydrolytic activity of the enzyme, even at a lower temperature (25°C; table 2). The preferential hydration of proteins that DMSO provokes at low concentrations is transposed to preferential interaction at high temperatures and high concentrations, with the consequent protein unfolding, and this phenomenon was not overcome with the insoluble preparation of αRβGl (table 2) [Arakawa et al., 2007]. Considering the instability generated by the increment of cosol-

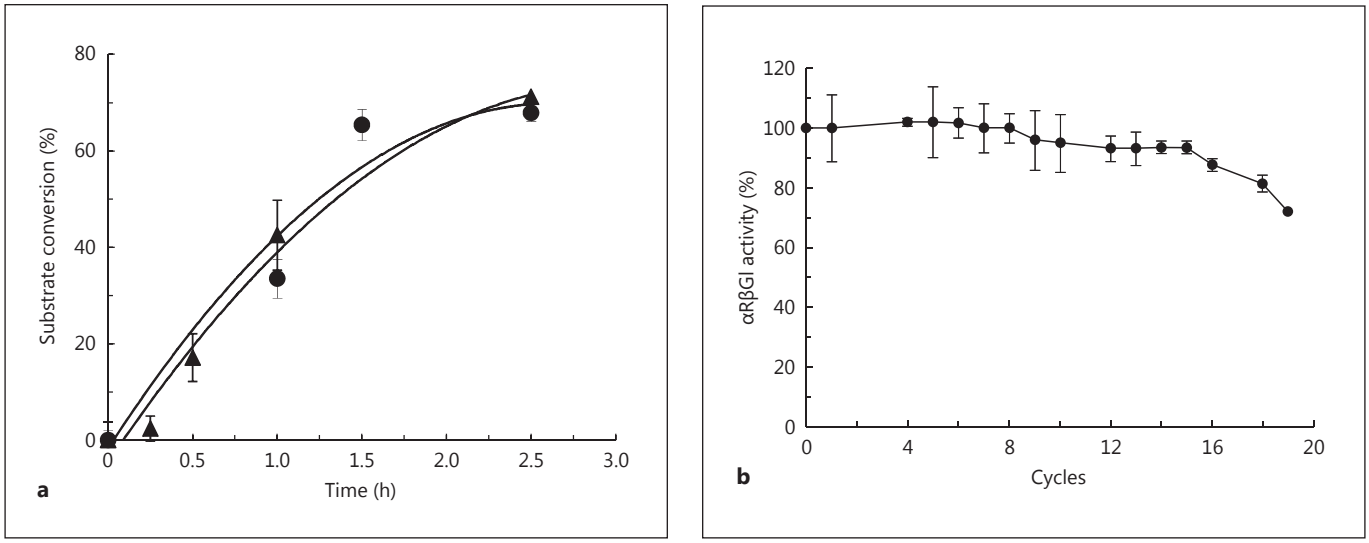

Fig. 4. Enzymatic production of hesperetin with αRβGl Ag-G derivative in a reaction medium containing 0.52 mM of hesperidin and 10% v/v DMSO at pH 5 and 60°C. **a** Reaction courses catalyzed by soluble (▲) and immobilized (●) enzyme. **b** Reusability of the biocatalyst preparation assessed in 20 cycles of the 2-hour reaction. The 100% activity corresponds to 0.72 U/ml and 0.55 U/g for soluble and immobilized enzyme, respectively.

vent concentration, the temperature was raised up to 60°C in a medium with 10% v/v DMSO. This system presented a higher productivity (3.36 μmol h^{-1} g^{-1}), slightly elevated compared to the productivity obtained at 50°C.

Production of Hesperetin

In this context, high concentrations of the cosolvent yielded a higher concentration of hesperetin, but a direct trade-off was established between production, productivity and enzyme destabilization. Hence, the process of hesperidin biotransformation was carried out using the free form and the Ag-G αRβGl in a medium containing 10% v/v DMSO, at 60°C. Although the time-course biotransformation showed a maximum substrate conversion of around 68% after 2–2.5 h for both soluble and immobilized enzyme, the Ag-G αRβGl preparation allowed the reutilization of the catalyst (fig. 4). Twenty cycles of hesperidin hydrolysis of 2 h at 60°C were performed with the immobilized enzyme. The productivity was constant after 15 cycles of reutilization (2.96 ± 0.42 μmol^{-1} h^{-1} g^{-1}) and it only dropped by ~20% after 20 operational cycles (fig. 4b).

Most of the reported enzymatic systems for hesperidin hydrolysis were performed using soluble biocatalysts (table 3). The hesperetin concentration yielded by the Ag-G αRβGl system was around 3.5 times higher than the system catalyzed by pectinase 62L (a bulk enzyme preparation), and 16-fold lower than a heterogeneous system that used supersaturated suspensions of hesperidin (16 mM) as the substrate. However, the specific productivities were ~25- and 37-fold lower, respectively, than the specific productivity found for the system herein presented (table 3) [Mandalari et al., 2006; Scaroni et al., 2002]. Moreover, an additional advantage of the reaction catalyzed by Ag-G αRβGl, besides the possibility of recycling it, is that it also coproduced another highly valuable product, the disaccharide rutinose. This is in contrast to the sequential enzymatic mechanisms of deglycosylation involving two monoglycosidases, where the aglycone productivity becomes a more complex biotransformation composed of five chemical species (the substrate, rhamnose, glucose, the glucosylated aglycone and the aglycone) [Orrillo et al., 2007; Scaroni et al., 2002; Spagna et al., 2002]. Recently, a one-step process for rutin deglycosylation using a thermophile promiscuous β-glucosidase and releasing quercetin and rutinose was also reported. In such a system, the specific productivity of quercetin was lower than the system herein described for hesperidin (table 3) [Nam et al., 2012].

Concluding Remarks

An immobilized αRβGl preparation was developed for hesperetin production from a low-cost byproduct of the citrus industry (hesperidin). The immobilization was op-

Table 3. Comparison of different systems for the enzymatic deglycosylation of three flavonoid glycosides

Flavonoid	Substrate concentration, mM	Enzyme	Substrate conversion, %	Specific productivity[a], mmol h^{-1} g^{-1}	References
Rutin	10	promiscuous β-glucosidase	65	1.04	[Nam et al., 2012]
Naringin	172	rhamnosidase glucosidase	80	28.21	[Ellenrieder et al., 1998]
	1.7	rhamnosidase	91	6.45[b]	[Chang et al., 2011]
Hesperidin	16	rhamnosidase glucosidase	35	0.09	[Scaroni et al., 2002]
	0.2	pectinase 62L	48	0.06	[Mandalari et al., 2006]
	0.52	αRβGl	64.6	2.40	this work

[a] Specific productivity was defined as the aglycone produced in mmol per hour per gram of protein. In the case of αRβGl it was estimated per gram of protein contained in the support.
[b] Production of the intermediate compound prunin (glucosylated naringenin).

timized rendering a highly stable biocatalyst able to perform the biotransformation in harsh conditions (10% v/v DMSO and 60°C). The heterogeneous catalyst, working in batch mode for 20 cycles, only lost 20% activity and reached specific productivity values of 2.40 mmol h^{-1} g^{-1}. Hence, a promising process for the hydrolysis of hesperidin has emerged, which could also be applied for other 7-*O*-rutinosilated flavonoids.

Experimental Procedures

Materials

Agarose 10 BCL was purchased from Agarose Bead Technologies (Madrid, Spain). Cyanogen bromide-activated Sepharose 4B (Ag-CB) was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Epichlorohydrin, ethylenediamine, PEI (MW 25 kDa), glutaraldehyde 25% w/v, DMSO, hesperidin, hesperetin and hesperidin methyl chalcone were purchased from Sigma (St. Louis, Mo., USA). All other chemicals were of analytical grade.

Strain, Growth Conditions and Protein Purification

Acremonium sp. DSM24697 was cultured using hesperidin as the carbon source and the enzyme was purified following the procedure previously described by Mazzaferro et al. [2010].

Activation of Agarose 10 BCL with Glyoxyl Groups (Ag-G)

This matrix was prepared by reaction of hydroxyl groups forming the agarose 10 BCL to either glycidol or epichlorohydrin. Agarose was activated with glycidol according to the protocol described by Guisán [1988], while to activate the agarose with epichlorohydrin, 10 g of agarose 10 BCL were suspended in 44 ml of water, 16 ml of acetone, 3.28 g of NaOH, 0.2 g of $NaBH_4$ and 11 ml of epichlorohydrin [Mateo et al., 2010]. The suspension was stirred mildly for 16 h and washed with an excess of water. The epoxy groups were hydrolyzed in the presence of 0.5 M of sulfuric acid (12 h, 25°C), resulting in a surface activated with vicinal diols that would finally be oxidized with sodium periodate. The quantification of glyoxyl groups on the resulting matrices was performed by determining the not consumed periodate after the oxidation step. The remaining periodate was determined with a 10% KI solution under alkaline conditions.

Agarose Activated with Primary Amine Groups (Ag-A)

Monoaminoethyl-N-ethyl-agarose was prepared from glyoxyl agarose according to Fernandez-Lafuente et al. [1993].

Agarose Activated with PEI (Ag-PEI)

The glyoxyl-agarose 4 BCL support was coated with PEI of average molecular weight 25 kDa. The support (5 g) was suspended in 50 ml of 10% w/v PEI at pH 11.0. The suspension was kept under mild stirring at 25°C for 2 h. Then, the support was reduced by adding solid sodium borohydride to a final concentration of 10 mg/ml and was left under mild stirring for 30 min at 4°C. Finally, the reduced suspensions were filtered and successively washed with an excess of distilled water and stored at 4°C.

Metal-Chelated Agarose

Ag-Ni^{2+} was prepared as previously described by Mateo et al. [2010]. Briefly, the epoxy groups of glyoxyl-agarose activated with epichlorohydrin, as described above, were partially hydrolyzed with 0.1 M of H_2SO_4 at pH 2.0 for 8 h. The remaining epoxy groups were incubated with 0.1 M of sodium iminodiacetate at pH 11.0 for 24 h at 25°C. Finally, the support was incubated with 0.23 M of $NiCl_2$ at pH 7.0 for 24 h at 25°C.

αRβGl Immobilization on Different Agarose-Type Matrices

Ten milliliters of enzyme solution (0.14 U/ml) at the indicated pH and conditions were mixed with 1 g of different activated supports. The immobilization course and the expressed activities were monitored measuring the enzyme activity in the supernatant and the suspension at different time points. Under all im-

mobilization conditions, a blank assay was carried out with inert agarose.

The conditions used to immobilize αRβGl on Ag-CB, Ag-A, Ag-PEI and Ag-Ni^{2+} were 25 mM of phosphate buffer at pH 7 and 25°C. In the case of immobilization on Ag-G, the immobilization needed to be carried out in 100 mM of sodium carbonate (pH 10.0) for 24 h at 25°C. These latter derivatives were finally reduced using 1 mg/ml of $NaBH_4$ solution to turn the reversible Schiff's base into an irreversible secondary amine. αRβGl immobilized on Ag-A and Ag-PEI were further cross-linked using glutaraldehyde. One gram of the immobilized enzyme preparation was suspended in 10 ml of 0.5% v/v glutaraldehyde in 25 mM of sodium phosphate buffer, pH 7.0. The suspension was kept under mild stirring at 25°C for 1 h. Then, the cross-linked derivative was filtered and washed exhaustively with 25 mM of sodium phosphate buffer at pH 7.0.

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

$$YI = \frac{A_{loaded} - A_{unbound}}{A_{loaded}} \times 100 \quad (1)$$

$$EI = \frac{A_{beads}}{A_{loaded} - A_{unbound}} \times 100 \quad (2)$$

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.

Enzymatic Activity Assays

αRβGl activity was quantified by measuring the increase in absorbance at 323 nm caused by the release of hesperetin during the hydrolysis of the substrate (0.1 mM hesperidin in 50 mM Tris-HCl buffer, pH 8.0) [Mazzaferro and Breccia, 2012]. The reactions were performed under continuous magnetic stirring at 50°C. One unit of αRβGl was defined as the amount of enzyme required to release 1 μmol of hesperetin per minute.

Stability Assays

The stability of different enzyme derivatives was assessed by incubating the preparations with 50% v/v DMSO solution at different temperatures. Samples of the suspension were withdrawn at different times, and residual activity was expressed as a percentage of the initial activity. The inactivation rate followed first-order kinetics; therefore, a nonlinear fitting using a single exponential decay model was performed [Yoon and McKenzie, 2005]:

$$[A]_t = [A]_O \exp(-kt) \quad (3)$$

where $[A]_t$ is the percentage enzyme activity at time t (h), $[A]_O$ is the initial percentage activity, and k the first order rate constant (h^{-1}). The half-life ($t_{1/2}$) of the preparation was calculated as 0.693/k as an index of stability.

Production of Hesperetin

The process of hesperetin production was evaluated using the selected immobilized preparation in different reaction media. Substrate concentration was assessed in the range of 0.50–15 mM of hesperidin. The effect of solvent concentration and temperature was performed in solutions between 10 and 70% v/v DMSO in 50 mM of sodium citrate at pH 5.0, and between 25 and 60°C, respectively. The quantification of the rendered hesperetin was performed using the method recently reported by Mazzaferro and Breccia [2012].

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