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Biosynthesis of an antiviral compound using a stabilized phosphopentomutase by multipoint covalent immobilization

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ABSTRACT

Ribavirin is a synthetic guanosine analogue with a broad-spectrum of antiviral activity. It is clinically effective against several viruses, such as respiratory syncytial virus, several hemorrhagic fever viruses and HCV when combined with pegylated interferon-α. Phosphopentomutase (PPM) catalyzes the transfer of intramolecular phosphate (from C1 to C5) on ribose, and is involved in pentose phosphate pathway and in purine metabolism. Reactions catalyzed by this enzyme are useful for nucleoside analogues production. However, out of its natural environment PPM is unstable and its stability is affected by parameters such as pH and temperature. Therefore, to irreversibly immobilize this enzyme, it needs to be stabilized. In this work, PPM from *Escherichia coli* ATCC 4157 was overexpressed, purified, stabilized at alkaline pH and immobilized on several supports. The activity of different additives as stabilizing agents was evaluated, and the best result was found using 10% (v/v) glycerol. Under this condition, PPM maintained 86% of its initial activity at pH 10 after 18 h incubation, which allowed further covalent immobilization of this enzyme on glyoxyl-agarose with a high yield.

This is the first time that PPM has been immobilized by multipoint covalent attachment on glyoxyl support, this derivative being able to biosynthesize ribavirin from α-D-ribose-5-phosphate.

1. Introduction

Nucleoside analogues are the most used effective class of antiviral agents. There are more than twenty drugs approved for treatment of viral diseases, and some of them are being tested in clinical trials (Ayoub and Keeffe, 2011; Shah et al., 2013). In particular, ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) is a purine nucleoside analogue that has shown a broad-spectrum activity against a variety of DNA and RNA viruses (Graci and Cameron, 2006), mainly including respiratory syncytial virus (Xu et al., 2004) and Lassa fever virus (Ölschläger et al., 2011), in addition to its combination with PEG-interferon, a standard therapy for chronic Hepatitis C in clinical situations (Brillanti et al., 2011; Feld et al., 2010; Thomas et al., 2012). Moreover, this molecule can be used as prodrug to achieve new compounds with a wide range of action and fewer side effects (Dong et al., 2013; Guo et al., 2015). This kind of compound is mainly synthesized by chemical methods that require organic solvents, multiple reaction steps and the removal of protective groups, yielding undesirable racemic mixtures (Pérez-Castro et al., 2007), decreasing the conversion rate and hindering product purification. Biocatalysis emerged to overcome some of these drawbacks because of its high catalytic efficiency, inherent selectivity and simple downstream processing. In addition, biotransformations take place under very mild conditions and offer environmentally clean technologies (De Benedetti et al., 2015; Rivero et al., 2012). Hence, the biosynthesis of these compounds can be performed stereoselectively by enzymes (Bzowska et al., 2000).

Phosphomutases (PPMs) are enzymes that rearrange the position of phosphate within a substrate molecule through either intra- or intermolecular phosphoryl transfer (Panosian et al., 2011). Bacterial PPMs (EC 5.4.2.7) transfer the phosphate group from position C5 of α-D-ribose-5-phosphate (R5P) to position C1, yielding α-D-ribose-1-phos-

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phate (R1P), which bridges glucose metabolism and RNA biosynthesis (Tozzi et al., 2006). R1P serves as a substrate for purine or pyrimidine nucleoside phosphorylases (PNP or PyNP) that accept a free base as a second substrate to displace the phosphate and form a nucleoside (Rivero et al., 2015).

Reactions catalyzed by these enzymes are useful for nucleoside analogues production; however, enzymes are unstable out of their natural environments, and parameters such as pH and temperature are key factors in stability (Homaei et al., 2013). Therefore, the stabilization mechanism represents an important issue in enzyme development for a possible industrial application. The low enzyme stability has been overcome by using genetic engineering strategies (Opperman and Reetz, 2010), applying chemical modifications (Xue et al., 2010), adding stabilizing agents (Nascimento et al., 2010; Santagapita et al., 2011) or using different immobilization mechanisms (Hernandez and Fernandez-Lafuente, 2011; Mateo et al., 2007). Particularly, immobilization by multipoint covalent attachment has been described as the most interesting approach to stabilize enzymes because it enables their continuous use and recycling in flow and batch reactors, respectively (López-Gallego et al., 2005; Ramírez Tapias et al., 2016). The immobilization of enzymes on highly activated glyoxyl supports under alkaline conditions promotes the multipoint covalent attachment of unprotonated ε-NH_2 groups of the surface lysine with the aldehyde groups at the support surface, forming Schiffs bases that are mildly reduced to turn them into irreversible protein-surface bonds. This immobilization process has revealed to be extremely efficient to stabilize many industrial enzymes (Bolivar et al., 2006; Fernández-Lorente et al., 2015). However, there are many enzymes that cannot be immobilized through this methodology because they become inactive under alkaline conditions. However, the addition of salts, polyols, dextran, sugars and other molecules is one of the most successful solutions to increase the enzyme stability under drastic conditions such as extreme pH values (Kaushik and Bhat, 2003; Ó'Fágáin, 2003; Castro, 2000).

Herein we report the stabilization of soluble PPM from *E. coli* under alkaline conditions using different additives, allowing for the first time its immobilization on glyoxyl supports. This kind of immobilization has revealed to be the most stabilizing one for PPM, resulting in immobilized PPM significantly more stable than its soluble and other immobilized counterparts. The obtained derivative was employed in the biosynthesis of ribavirin using an environmentally friendly methodology.

2. Materials and methods

2.1. Materials

Adenine, adenosine, 1,2,4-triazole-3-carboxamide (TCA), α-D-ribose-1-phosphate (R1P), D-ribose-5-phosphate (R5P), α-D-glucose 1,6-bisphosphate, purine nucleoside phosphorylase (PNP), ethylenediamine (EDA), polyethylene glycol (PEG) (Mw 4000), glycerol, dextran (Mw 1500) and trehalose were purchased from Sigma Chem. Co (St. Louis, USA). Cyanogen bromide (CNBr) activated Sepharose beads and agarose 10BCL were purchased from GE-Healthcare (Uppsala, Sweden). Culture media compounds were obtained from Britania S.A. (Argentina). The HPLC grade solvents used were from Sintorgan S.A. (Argentina).

2.2. Cloning and PPM expression

Genomic DNA for *E. coli* ATCC 4157 was isolated by using DNA isolation kit from QIAGEN (Hilden, Germany) and the gene encoding enzyme PPM was amplified by polymerase chain reaction (PCR) using 5′TTTTAGATCTCATATGGAACGTGCA-3′ as forward primer and 5′ATATGGCAAAGCCATGTTCCTCGAG-3′ as reverse primer with NdeI and XhoI restriction endonucleases sites, respectively. PCR reactions were performed using 1 U per tube of Taq DNA polymerase (Promega, USA), 3 mM $MgCl_2$, 2.5 μL DMSO, 0.2 mM dNTPs 0.2 μM for each primer, and PCR quality water added to a final volume of 50 μL per tube. The PCR product was linked with pET-22b (+) plasmid (Novagen, Germany) and transformed into *E. coli* strain BL21 (DE3) from Stratagene (California, USA). Cells were grown in LB supplemented with 100 μg/mL ampicillin at 37 °C and 200 rpm until the culture reached a density of 0.6 units at 600 nm. Protein expression was induced by addition of 0.1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) for different times. Pellets were harvested by centrifugation at 6000 rpm for 20 min and suspended in phosphate buffer (25 mM, pH 7). Cells were disrupted by sonication for 10 min at 30 kHz in ice water (Vibra Cell, USA) and clarified by centrifugation at 11,000 rpm for 30 min at 4 °C.

2.3. PPM purification

The enzyme was purified from the supernatant by affinity chromatography using a HisTrap HP column (GE). Sodium chloride (150 mM) and imidazole (10 mM) were added to the enzymatic solution and used to equilibrate the column. Nonspecifically bound proteins were eluted by adding phosphate buffer (25 mM, pH 7) supplemented with 150 mM NaCl and 25 mM imidazole. The purified protein was eluted by adding phosphate buffer (25 mM, pH 7) supplemented with 150 mM NaCl and 300 mM imidazole. Protein concentration was determined by Bradford method (Bradford, 1976) and purification of the protein was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using Coomassie Brilliant Blue staining.

2.4. PPM activity

2.4.1. Standard reaction

The enzymatic activity was evaluated by measuring adenosine biosynthesis from R5P and adenine via R1P in the presence of commercial PNP previously immobilized in CNBr activated agarose (Mateo et al., 2005). The standard reaction was carried out at 25 °C using 0.5 mM adenine, 0.1 mM R5P, 100 μL PNP- CNBr suspension (0.01 mg of protein) and 0.05 mg of soluble PPM in 1 mL of phosphate buffer (25 mM, pH 7). The biosynthesis of adenosine was detected and quantified by high-performance liquid chromatography (HPLC).

2.4.2. Reaction optimization

The effect of manganese (II) ion (0.05 mM) and different buffers such as sodium phosphate and tris-(hydroxymethyl)aminomethane–HCl (Tris–HCl) were evaluated. Reactions were performed at different times under previously described standard conditions.

Moreover, the addition of several α-D-glucose 1,6-bisphosphate amounts (0, 1 and 3 μM) and different manganese ion concentrations (0.05, 0.10 and 0.15 mM) were evaluated at 1 h under the same reaction conditions.

2.4.3. Effect of additives on PPM stability

Samples containing PPM solution were treated with water solutions (20%, v/v) of PEG (Mw 4000), glycerol, dextran (Mw 1500) and trehalose. The preparations were incubated at 4 °C for 18 h in carbonate buffer (100 mM, pH 10). The enzymatic activity was assayed at different times under the conditions described above. All the experiments were carried out by triplicate. After additive selection, different glycerol concentrations such as 5%, 10%, 20% and 40% (v/v) were evaluated.

2.5. PPM immobilization

Previously purified PPM was immobilized on agarose activated with cyanogen bromide (CNBr-Ag), on monoaminoethyl-N-aminoethyl agarose (MANAE-Ag) or MANAE-glutaraldehyde (MANAE-G) (Pessela et al.,

2006) and glyoxyl agarose (Gx-Ag) (Mateo et al., 2006).

Ten mL of PPM solution (0.7 mg/mL) were added to 20 mL of sodium phosphate buffer (10 mM, pH 7). Then, 1 g of the CNBr-Ag, MANAE-Ag or MANAE-G support was added. The mixture was stirred mildly for 2 h, at 4 °C for CNBr immobilization and at 25 °C for MANAE immobilization. When the CNBr-Ag support was used, after the supernatant was removed, 30 mL of ethanolamine (1 M, pH 8) were added for 2 h to block the cyanogen bromide groups on the support. Finally, the derivative was washed with sodium phosphate buffer (25 mM, pH 7). For the preparation of the multipoint covalent immobilized catalysts, a 10 mL of PPM enzyme and glycerol solution (10%, v/v) was prepared in sodium bicarbonate buffer (100 mM, pH 10). The obtained solution was stirred mildly with 1 g of Gx-Ag support for 8 h at 4 and 25 °C. Afterwards, the derivative suspension was reduced with 1 mg/mL sodium borohydride for 30 min.

2.6. Thermal stability of PPM derivatives

To study the thermal stability, the obtained derivatives were incubated at 50 °C for different times. In this sense, aliquots of the suspensions were withdrawn and their residual activities were tested as described previously.

2.7. Structural modeling and surface analysis

The structural model was built using the structure of PPM from *Bacillus cereus* (PDB 3UO0), the Swiss-Model homology-modeling server and the alignment mode (Biasini et al., 2014). The quality of the model (Z-score and QMEAN) met the standards recommended by the server. The electrostatic surface potential and the surface local flexibility were calculated with Bluues and COREX/BEST servers, respectively. The protein model and surface analysis were visualized using PyMol 0.99 developed by DeLano Scientific LLC (San Francisco, CA, USA).

2.8. Ribavirin biosynthesis

The biosynthesis of ribavirin was carried out at different times in 1 mL of sodium phosphate buffer (25 mM, pH 7) containing 5 mM TCA and 1 mM R5P, 0.1 mM manganese (II), 1 μM α-D-glucose 1,6-bisphosphate, 500 μL PNP- CNBr suspension (0.05 mg of protein) and 250 μL PPM-Gx derivative (0.25 mg of protein) at 25 °C and 200 rpm.

2.9. Green chemistry parameters

Green chemistry parameters of the aforementioned bioprocesses were calculated as previously described (Cappa et al., 2014). The Environmental factor (E-Factor) is a measurement of the industrial environmental impact. Carbon efficiency (C-Efficiency) and atom economy (A-Economy) are designed as parameters to evaluate the efficiency of synthetic reactions.

2.10. Analytical methods

Nucleoside biosynthesis reactions were monitored by HPLC (Gilson) equipped with a UV detector (UV/Vis 156, Gilson) using a Nucleodur 100-5 C18 column (5 μm, 125 mm x 5 mm). For adenosine biosynthesis, an isocratic mobile phase of water/methanol (95:5, v/v) and a flow rate of 1.2 mL/min were used and evaluated at 254 nm. Adenine and adenosine retention times were 4.3 and 6.2 min, respectively. Ribavirin bioconversion was quantitatively monitored at 225 nm using a Phenomenex Luna® C-18 column (5 μm, 250 mm x 4.6 mm). An isocratic mobile phase (100% water) was used at a flow of 1.2 mL/min. The retention times of substrates and products were 4.1 and 7.0 min for TCA and ribavirin, respectively.

Furthermore, product identification was performed by MS-HPLC under the above-mentioned conditions (ribavirin; M+: 245.1) using an LCQ-DECAXP4 Thermo Finnigan spectrometer with the electron spray ionization (ESI) method and one ion trap detector.

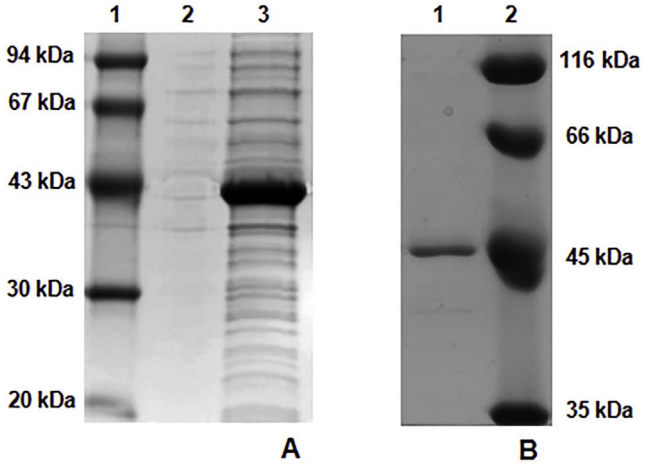

Fig. 1. SDS-PAGE of recombinant PPM from *E. coli.* A) PPM expression. Lane 1: protein marker, lane 2: total proteins before induction, lane 3: soluble fraction after 3 h of induction. B) PPM purification using HisTrap HP column. Lane 1: purified PPM, lane 2: protein marker.

3. Results and discussion

3.1. PPM expression and purification

PPM gene was amplified from *E. coli* genomic DNA and cloned into plasmid pET-22b (+). The cloned sequence showed 100% identity to the *E. coli* phosphopentomutase (GI:999847787). The plasmid encoding PPM gene tagged with polyhistidine tag was transformed into *E. coli* and His-PPM was expressed by induction with IPTG and purified by IMAC chromatography. After 3 h of induction, the protein mainly appeared in the soluble fraction after sonication, showing that this enzyme does not form inclusion bodies (Fig. 1A). Higher induction times increased protein degradation (data not shown). After purification, 0.7 mg/mL of pure PPM was obtained, which presented a molecular weight of 43 kDa, according to the one previously reported by gradient SDS-PAGE (Fig. 1B) (Leer and Hammer-Jespersen, 1975).

3.2. Effect of different additives on PPM activity

Enzymatic activity was evaluated for adenosine biosynthesis using a multienzymatic system based on soluble PPM and previously stabilized PNP (PNP-CNBr) activity (Fig. 2). This bi-enzyme system is able to biosynthesize adenosine from R5P, coupling a phosphate transfer reaction with the enzymatic addition of a nitrogenated base to the corresponding phosphorylated ribose. Different parameters such as buffer, manganese (II) ion and α-D-glucose 1,6-bisphosphate addition were evaluated to improve the reaction yield. The effect of different buffers (phosphate and Tris-HCl) on the performance of PPM/PNP system was tested first (Fig. 3A), since it has been reported that PPM activity is affected by phosphate buffer (Hammer-Jespersen and Munch-Petersem, 1970) but in turn, PNP requires traces of this ion (Xie et al., 2011). No significant differences in adenosine yield between the tested buffers were found. On the other hand, manganese (II) is a structural member of the PPM active site. Previous studies of active site architecture of prokaryotic PPMs by crystallographic techniques revealed the presence of two ions in coordination with amino acidic residues playing a key role in the PPM catalytic mechanism (Panosian et al., 2011). In fact, these results have been confirmed since PPM activity significantly increased when manganese (II) was added to the reaction media (Fig. 3A). Moreover, several manganese (II) concentrations were evaluated, 0.1 mM being the most favorable condition

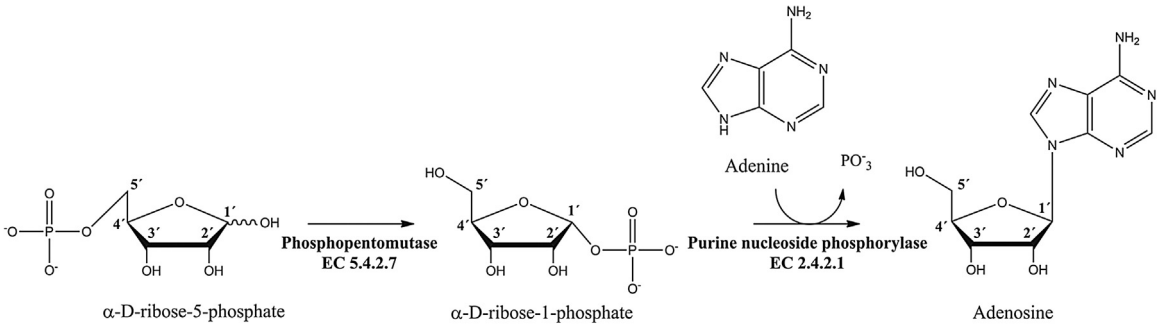

Fig. 2. Biosynthesis of adenosine by a multienzymatic system. A standard reaction, where adenine and R5P were used as substrates, was assayed to evaluate soluble PPM activity.

(Fig. 3B).

Furthermore, phosphorylation of PPM requires an activation step triggered by α-D-glucose 1,6-bisphosphate. In this case, PPM activity increased 7-fold when 1 μM inductor was added to the reaction. However, no significant differences were observed at higher concentrations of α-D-glucose 1,6-bisphosphate (data not shown). This activation is one requirement for the mechanistic divergence between PPM and alkaline phosphatase (Iverson et al., 2012).

3.3. PPM stabilization under alkaline conditions

The immobilization of PPM on glyoxyl (aldehyde) activated supports requires the incubation of the enzyme at pH 10. To test the effect of the pH on the stability of PPM, the soluble enzyme was incubated at different pH values. The enzyme incubated at pH 7 maintained around 90% activity for more than 24 h. However, the same enzyme lost 70% of its initial activity after 18 h of incubation at pH 10. This shows that the stability of the enzyme is extremely low at alkaline pH, a condition necessary to perform the multipoint covalent immobilization on glyoxyl support.

The protective effect of additives such as PEG, trehalose and glycerol on the enzymatic activity under drastic conditions is very well known, and the protection degree frequently depends on the additive concentration (Castellanos et al., 2003; Salahas et al., 1997). Moreover, it has been widely reported that polyols possess a chemical chaperone-like activity capable of stabilizing the native conformation of enzymes in the presence of several kinds of stress, inhibiting denaturalization (Zancan and Sola-Penna, 2005). Thus, different additives were added to the enzyme solution to study their effect on the enzyme stability. Fig. 4A shows that glycerol clearly stabilizes soluble PPM at pH 10 since this enzyme retained 86% of its initial activity after incubation for 18 h, whereas the same enzyme maintained only 30% of its initial activity when additives were not included in the soluble preparation. Accordingly, this polyol was selected as the best protective additive and its stabilizing effect was evaluated by varying the glycerol concentration. Since the enzyme practically retained its initial activity after 18 h-incubation at pH 10, 10% (v/v) glycerol was selected as the best additive concentration (Fig. 4B). This result allowed for the first time to immobilize PPM through glyoxyl chemistry at alkaline pH values.

3.4. PPM immobilization

PPM was immobilized at pH 10 on agarose activated with glyoxyl groups (Gx-Ag) at two different temperatures and in the presence of 10% (v/v) glycerol as stabilizing additive. Additionally, it was immobilized at pH 7 on agarose activated with cyanogens (CNBr-Ag), positively charged amine groups (MANAE-Ag) and glutaraldehyde groups (MANAE-G). Fig. 5 shows the immobilization courses of PPM on different agarose supports. The immobilization of PPM on MANAE-Ag, MANAE-G and Gx-Ag (25 °C) was faster than on CNBr-Ag and Gx-Ag (4 °C). Retained specific activity after the immobilization process is shown in Table 1, and it is noteworthy that expressed activity after immobilization under alkaline conditions was due to the addition of glycerol 10% (v/v) as stabilizing agent.

3.5. Thermal stability of PPM derivatives

To evaluate the effect of the immobilization on enzyme stabilization, we incubated the different immobilized preparations of PPM at 50 °C and monitored their residual activity at different times. PPM immobilized on Gx-Ag support was the most stable preparation regardless of the immobilization temperature. Both insoluble derivatives maintained 100% of their initial activity after 16 h at 50 °C, whereas PPM immobilized on MANAE-Ag lost more than 50% of its initial activity under the same conditions. The least stable insoluble preparation was the PPM immobilized on CNBr-Ag, which was completely inactive after only 4 h incubation at 50 °C (Fig. 6).These results indicate that the thermal stability achieved with the glyoxyl chemistry was possibly due to a multipoint covalent attachment between the PPM and the support surface that may prevent the protein denaturalization even under drastic conditions. This high thermal stability encouraged us to use this heterogeneous biocatalyst in the biosynthesis of nucleo-

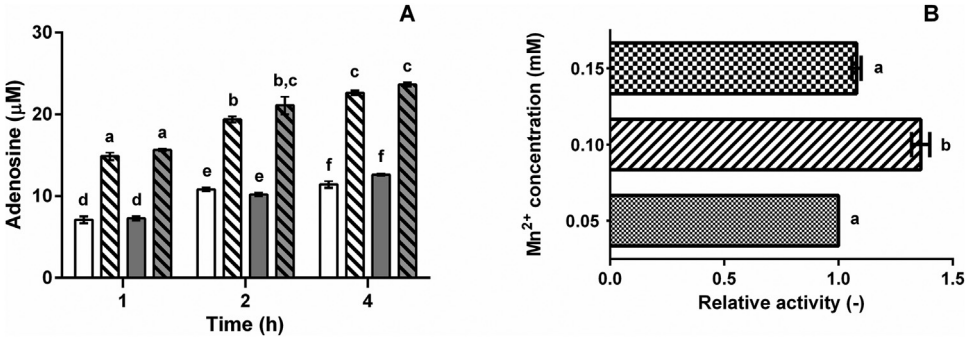

Fig. 3. Effect of buffer and manganese (II) on PPM activity. A) Sodium phosphate (white) and Tris-HCl (gray) buffers were evaluated without (open bars) or with manganese (striped bars) for adenosine biosynthesis. B) Different manganese (II) ion concentrations were evaluated. All reactions were performed three times and according to Duncan's test for mean data comparison, significant differences among treatments named with different letters (p-value < 0.05) were observed.

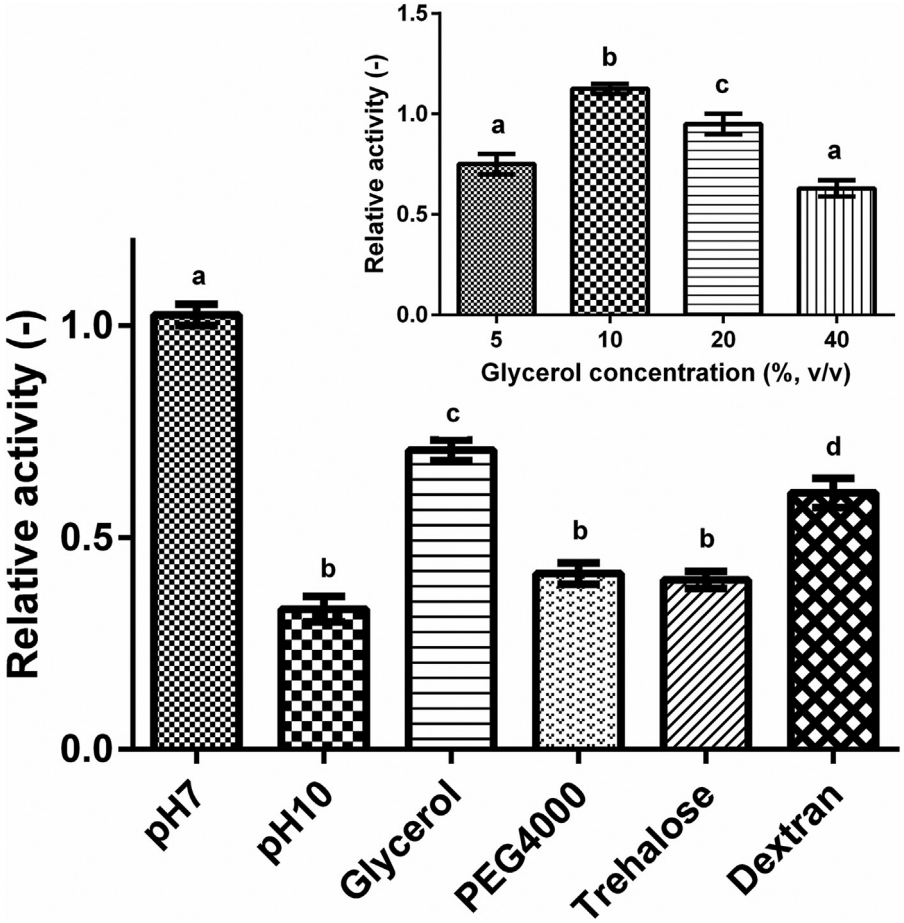

Fig. 4. Enzyme stability at alkaline pH. A) Different additives (20%, v/v) were evaluated for PPM stabilization. B) Effect of glycerol concentrations. All reactions were performed three times and according to Duncan's test for mean data comparison, significant differences among treatments named with different letters (p-value < 0.05) were observed.

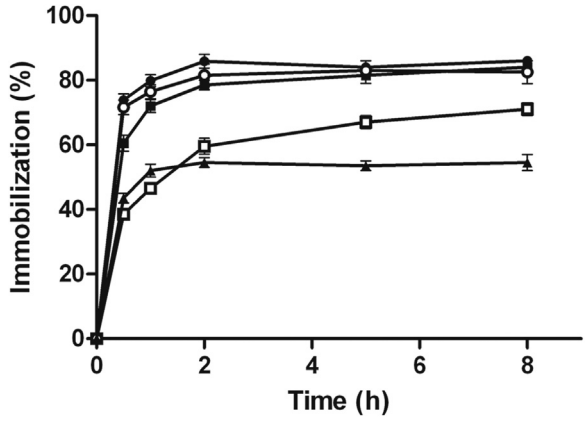

Fig. 5. Immobilization courses of PPM on different supports. MANAE-Ag (●), MANAE-G (○), Gx-Ag at 25 °C (■), Gx-Ag at 4 °C (□) and CNBr-Ag (▲). All reactions were performed three times.

side analogues because in this work, the operating temperatures reported do not exceed 30 °C. Moreover, the improved thermal stability of the immobilized PPM could allow its reuse in successive cycles without significant loss of activity.

3.6. Structural modeling and surface analysis

3.6.1. Orientation versus activity

To explain the immobilization results at molecular level, we built a homology protein model based on the high identity (45%) of the PPM

Table 1
PPM immobilization on different supports.

	Immobilization yield (%)	Retained activity (%)	Specific activity (U/mg)[a]
Soluble PPM	–	–	2.00
CNBr-Ag	54	51	1.02
MANAE-Ag	86	93	1.86
MANAE-G	82	88	1.76
Gx-Ag (25 °C)	84	78	1.56
Gx-Ag (4 °C)	71	86	1.72

[a] Enzyme activity (U) was calculated as μmol/h.

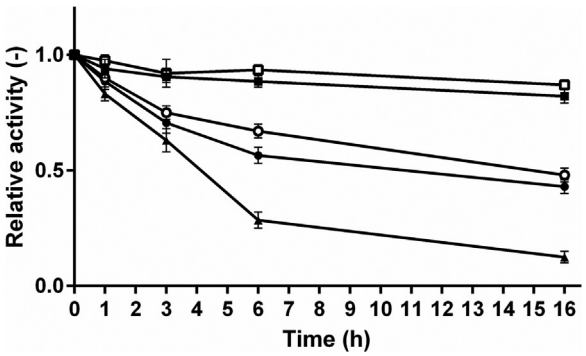

Fig. 6. Thermal inactivation of PPM derivatives at 50 °C. MANAE-Ag (●), MANAE-G (○), Gx-Ag at 25 °C (■), Gx-Ag at 4 °C (□) and CNBr-Ag (▲). All reactions were performed three times.

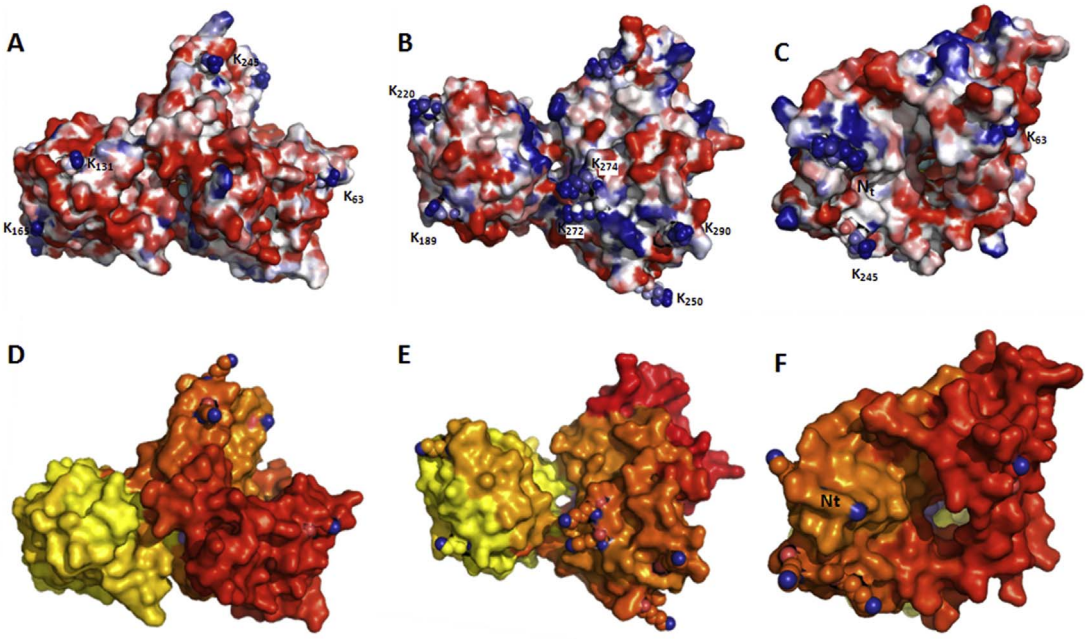

Fig. 7. Proposed orientation of PPM immobilized on different carriers based on *In silico* surface analysis of a PPM analogy model. A structural model of PPM was built based on sequence analogy (SWISS MODEL-Expasy) and using PDB: 3UO0 as template. The electrostatic surface potential (negative residues in red, positive residues in blue) of the PPM model was calculated by using Bluues server (A, B and C), while the local flexibility (color gradient between flexible residues in red and rigid residues in yellow) of this model was calculated by using COREX/BEST server (D, E and F). Images represent the PPM orientation when it was immobilized on MANAE-Ag (A and D), on Gx-Ag (B and E) and on CNBr-Ag (C and F). In all cases, the lysine residues with an apparent pKa $<$ 11.5 and with an exposed surface $>$ 50 Å^2 are represented as spheres. Inlet panel A depicts the active site of PPM where manganese ion (cyan spheres) and T98 and K259 (yellow sticks) are indicated. The figure was created with pymol 0.99 (DeLano, USA). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

from *E. coli* with the same enzyme from *Bacillus cereus* containing three manganese (II) atoms at its active site. The model, in concordance with the template, shows the catalytic threonine 98 and the lysine 259 involved in the enzyme activation triggered by the 1,6-biphosphate glucose (Iverson et al., 2012). This model was used to calculate the electrostatic surface potential of this enzyme to propose an orientation of PPM when it is immobilized on different supports. According to the surface analysis, the immobilization on MANAE-Ag likely takes place through the most acidic region (Fig. 7A), which presents a higher affinity for the positively charged amine groups on the carrier surface. On the other hand, the immobilization on Gx-Ag is driven by the reactivity and the density of lysine residues at the protein surface (López-Gallego et al., 2013). According to the model, we propose that PPM is immobilized on Gx-Ag through the region shown in Fig. 7B that displays six lysines (K189, K220, K250, K272, K274 and K290) fairly exposed to the media and with apparent pKa lower than 11.5. Such region clearly allows the nucleophilic attack on the aldehyde groups on the support surface under alkaline conditions (pH 10). Finally, we propose that the orientation of PPM on the CNBr-Ag surface occurs through the protein N-terminus (Fig. 7C), which involves a smaller and more flexible area of the protein surface compared to the orientations proposed for Gx-Ag and MANAE-Ag. In this orientation, the access to the active site seems to be partially blocked, which may explain the low specific activity of PPM after immobilization on CNBr-Ag (Table 1). On the contrary, the orientation of PPM on both MANAE-Ag and Gx-Ag seems not to interfere in the substrate access and the catalytic mechanism, supporting the higher specific activities for the corresponding immobilized derivatives (around 70% of immobilized activity) (Table 1).

3.6.2. Orientation versus stability

The orientation of the PPM immobilized on the different agarose supports also affects the thermal stability of the heterogeneous biocatalyst. Fig. 7 shows how different orientations provide different intensity in the covalent attachment between the protein and solid surface, and moreover the attachments occur in regions with different rigidity (Fig. 7D–F). In the first case, the immobilization of PPM on Gx-Ag seems to favor the formation of at least six irreversible covalent bonds after the reduction treatment, while in the case of CNBr-Ag the enzyme may be attached through the N-terminus, although K63 and K245 may eventually react with the cyanogen bromide groups, increasing the intensity of the covalent attachment. Nevertheless, both the low immobilization yield (Table 1) and low stability (Fig. 6) of the PPM immobilized on CNBr-Ag do not support the formation of additional covalent bonds with those exposed lysine residues (Fig. 7C). On the other hand, the covalent immobilization of PPM on MANAE-G activated with glutaraldehyde groups is primarily driven by an ionic absorption between the most acidic region of PPM and the positively charged groups on the carrier surface to further establish a covalent interaction between the glutaraldehyde groups and likely the lysines K63, K131, K165 and K245. This orientation resulted in a more stable immobilized protein than when it was immobilized on CNBr-Ag, but it was less stable than the same enzyme immobilized on Gx-Ag. According to the structural model and the surface analysis, the immobilization of PPM on Ag-MANAE, as well as the immobilization on CNBr-Ag, involves the flexible N-terminus domain (Fig. 7D and F), which seems to give lower stabilization factors unlike the immobilization on Gx-Ag where such flexible region was not involved and higher stabilization factors were achieved (Fig. 7E). Therefore, the rigidification of such flexible region, although mild, seems to limit the thermal stability of the immobilized protein. Those immobilization chemistries that do not involve such region will better guarantee the final stabilization of the heterogeneous biocatalyst.

3.7. Ribavirin biosynthesis

Previous studies have reported that nucleoside analogues can be successfully synthesized by using enzymes as catalysts (Wei et al., 2008; Yokozeki and Tsuji, 2000). The genes encoding for the enzymes involved in this biosynthesis pathways have been identified (Esipov

et al., 2002; Lee et al., 2001). However, these studies have focused on the cloning and expression of a single gene in order to assess enzymatic activity, and few have addressed the role of these enzymes in the biosynthesis of nucleoside analogues. In this work, ribavirin biosynthesis from TCA and R5P was carried out by using a one-pot system. The reaction was performed under the previously optimized conditions and using PPM optimally immobilized on Gx-Ag support in combination with PNP-CNBr as biocatalysts. This one-pot reaction yielded 150 μg of ribavirin in 3.5 h at 25 °C. Although in some papers it has been reported that ribavirin is obtained by biocatalysis, these methods are mainly based on the use of whole cells (De Benedetti et al., 2015; Rivero et al., 2015), which contain a large amount of active enzymes that promote the appearance of side products interfering with the purification of the product of interest. On the other hand, it has been reported that ribavirin may be obtained from natural nucleosides using an enzyme called PNP with similar yields to those found in this work (Konstantinova et al., 2004; Xie et al., 2011). However, these biotransformations are carried out at temperatures above 50 °C, and longer reaction times are required.

Therefore, this is the first time that ribavirin biosynthesis from simple sugars has been carried out by a stabilized multienzymatic heterogeneous biocatalyst, improving environmental sustainability compared to those reactions performed by whole cells. Just as previously reported, in the present work, E-Factor values for ribavirin biotransformation were lower than five (Rivero et al., 2015), suggesting mass utilization efficiency and a significant decrease of waste production. Furthermore, a C-Efficiency of 100% and an A-Economy of 72% were obtained. These parameters have been significantly enhanced with respect to those obtained using microorganisms, which imply a very positive effect on atom recovery and process efficiency. Finally, this developed multienzymatic biocatalyst could be evaluated in the biosynthesis of other nucleoside analogues with antiviral or antitumor ability, using R5P as starting substrate and allowing a biocatalytic process significantly cheaper than the same process from R1P.

4. Conclusions

Although enzymatic synthesis has several potential advantages, nucleoside analogues are still synthesized by chemical procedures on a large scale. The limitations for their practical application are mainly due to relatively low enzymatic activity and stability.

In this work, phosphopentomutase from *E. coli* was stabilized at alkaline pH by the addition of glycerol (10%, v/v) as stabilizing agent, which permitted–for the first time as far as we know–the immobilization of this enzyme on supports activated with glyoxyl groups at pH 10, obtaining excellent results in terms of immobilization yield and expressed activity after the immobilization process. Furthermore, this immobilization chemistry enables an improved thermal stability of PPM allowing its application in the biosynthesis of a commonly used antiviral compound. In this report, an efficient one-pot bioprocess using immobilized enzymes is described for the production of ribavirin. This process meets the requirements of high stability and short reaction times needed for low-cost production in a future preparative application.

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