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Coupling of Site-Directed Mutagenesis and Immobilization for the Rational Design of More Efficient Biocatalysts: The Case of Immobilized 3G3K PGA from *E. coli*

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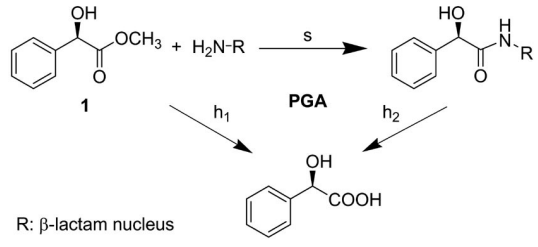
We have investigated the synthetic performance of the immobilized 3G3K mutant of the Penicillin G acylase (PGA) from *E. coli* obtained by site-directed mutagenesis. The 3G3K mutant, characterized by a tag consisting of three lysines alternating with three glycines at the end of the β-chain, has previously been reported to have a higher ratio for the rate of antibiotic synthesis and the rate of hydrolysis of the acylating agent (the vs/vh_1 value) than the wild-type enzyme. New immobilization studies have been carried out with the 3G3K mutant by using different glyoxyl supports (activated with aldehyde groups). The catalytic properties of the new immobilized preparations were tested in the synthesis of Cefamandole and Cefonicid by kinetically controlled *N*-acylation (*kcNa*). Compared with the commercial wild-type PGA, the 3G3K acylase immobilized on glyoxyl agarose showed higher synthetic performance (reaction rates and yields) in all the tested reactions.

Introduction

Penicillin G acylase (PGA; E.C. 3.5.1.11) is widely used in industrial processes for the semi-synthesis of β-lactam antibiotics,[1–4] as well as in the resolution of racemic mixtures, the synthesis of amides and for selective deprotection.[5–11]

The β-lactam antibiotics can be synthesized by the kinetically controlled *N*-acylation (*kcNa*) of the β-lactam nucleus. The yield depends on the balance of different reactions (Scheme 1) catalyzed by the same enzyme,[4,12–15] specifically, on the ratio between the rates of antibiotic synthesis (vs) and the acyl donor hydrolysis (vh_1). The vs/vh_1 ratio concerns the initial rates (before 20% of the acyl donor is converted) and defines the percentage of ester utilized for the synthetic process at the beginning of the reaction (when the hydrolysis of the acylation product, h_2, is negligible). A high value of the initial vs/vh_1 means that the acyl moiety is transferred with high efficiency to the nucleus and, therefore, that the acyl donor molar excess required to achieve complete acylation can be low. This parameter depends on the structure and the concentration of the β-lactam nucleus.[16]



Scheme 1.

The cost, the loss and/or the denaturation of the enzyme are the main limitations for its application on an industrial scale. To ensure enzyme stability, recovery and reuse, PGA from *E. coli* is routinely used after immobilization on solid supports.[1,17]

However, in spite of the advantages of this approach, the immobilization should not negatively interfere with the interaction of the substrates with the active centre and the conformational changes the enzyme undergoes therein.[18–20] Consequently, the influence of any structural change (i.e., a distortion of the amino acid residues involved in catalysis and/or in substrate binding) induced by immobilization cannot be underestimated. In fact, it has been reported that immobilization influences the catalytic properties of PGA,[21] depending on the type of matrix, the

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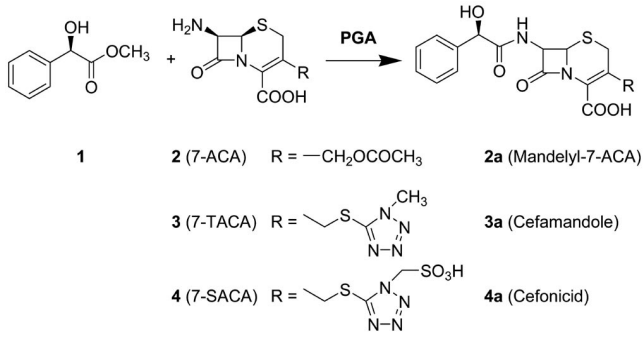
binding chemistry and the microenvironment created around the active site. The resulting effect can be crucial, particularly when the enzyme is immobilized with the active site near the support surface, because this can hamper the diffusion of substrates into the active site of the enzyme.

Depending on the immobilization procedure, enzymes can be immobilized by different mechanisms and, thus, with different outcomes. By using glyoxyl supports (activated with aldehyde groups) the immobilization proceeds via the formation of a Schiff base[22,23] between the protein's free amino groups (lysines) and the support's aldehyde groups. Therefore, the enzyme is immobilized through the areas bearing the highest density of lysines.[24] When it is not possible to further modulate the properties of the support to ensure a better diffusion of the substrates, the enzyme surface can be modified by site-directed mutagenesis. Site-directed mutagenesis has been shown to be a powerful tool for improving the catalytic properties and stability of several enzymes,[25,26] but can also be used to improve immobilization efficiency[27] or, as in the case of PGA, to induce a preferential orientation of the enzyme during the binding process by favouring the immobilization of the protein on the face opposite the active site.[28] In fact, in the case of glyoxyl agarose, when additional lysines were introduced onto the protein surface, a remarkable increase in enzyme stability was observed[29] as well as a better control of the immobilization process: the binding of the enzyme onto the support was forced through the lysine-rich region, thus leaving the active site fully accessible to the substrate.[30]

Recently, a new mutant of PGA, characterized by a basic and flexible tag of three lysines alternating with three glycines introduced at the C-terminus of the β-chain (on the side-face of the protein[31]), has been described.[32] Preliminary results demonstrated that this mutant (3G3K PGA), compared with the wild-type acylase, is characterized by a higher vs/vh_1 ratio in the *kcNa* of 7-aminocephalosporanic acid (7-ACA)[32] and maintains this property after immobilization. We hypothesized that the flexibility of the tag and the increased number of lysines probably induce the preferential and oriented immobilization of this PGA, leading the β-lactam substrate to freely access the active site of the immobilized enzyme.

The encouraging results obtained with the immobilized 3G3K PGA prompted us to extend our investigation to the evaluation of different enzyme preparations of this mutant as catalysts for the preparative synthesis of some cephalosporins. Thus, 3G3K PGA has been immobilized on glyoxyl agarose and glyoxyl Sepabeads, the surfaces of which differ in their hydro-/lipophilic nature. The most active biocatalyst was tested in the *kcNa* of different β-lactam nuclei (**2**–**4**) with (*R*)-mandelic acid methyl ester (**1**; see Scheme 2 and Table 3). The synthesis of 7-(1-hydroxy-1-phenylacetamido)-3-acetoxymethyl-Δ^3-cephem-4-carboxylic acid (**2a**, mandelyl-7-ACA) was considered because this compound can be used as a precursor of Cefamandole (**3a**) and Cefonicid (**4a**), as previously reported.[33,34] Additionally, the direct acylation of **3** and **4** with (*R*)-mandelic acid methyl ester (**1**) was performed to give **3a** and **4a** (Scheme 2). The results have been compared with those of the commercial wild-type PGA to test if the addition of the tag somehow affects the catalytic properties of the enzyme.



Scheme 2.

Results and Discussion

Immobilization

The commercial wild-type (wt) PGA was immobilized at 25 °C and pH 10 by following the standard procedure previously reported.[35] Under these conditions, about 90% of the loaded protein (13 IU/g) was immobilized on glyoxyl agarose by multipoint attachment with 73% of expressed activity (measured as a percentage of the offered activity to the support and expressed after immobilization; Table 1). Immobilization of 80 IU/g of the commercial wt PGA on glyoxyl agarose gave a 58% yield. For the 3G3K PGA, because of the poor stability of this enzyme,[32] the immobilization was performed at 4 °C and pH 9.5. The immobilization on glyoxyl agarose under these conditions was remarkable: in fact, 51% of the offered activity was bound with a 30% yield. Loading a higher amount of activity on glyoxyl agarose gave very similar results: in fact, a load of 100 IU of 3G3K PGA per gram of support afforded an immobilized preparation with a 30 IU/g activity (Table 1). The loss of about 50% of activity exactly corresponds to the decrease of activity caused by the poor stability of this enzyme at pH 9.5 and 4 °C, in accord with data previously reported.[32] With the aim of directly comparing the immobilized preparations of the commercial wt and the mutant PGA, the former was also immobilized at 4 °C and pH 9.5 (Table 1). The percentage of immobilized wt enzyme was negatively affected in comparison with the 3G3K, in agreement with previous data.[32] In fact, the percentage of immobilized commercial wt PGA was three-fold lower (Table 1).

When Sepabeads was used as the support using experimental conditions that guarantee the 3G3K activity (pH 9.5, 4 °C), the commercial PGA displayed all the immobilized activity, whereas a partial loss of activity was observed at 25 °C and pH 10 on the same support: 83% of the commercial wt PGA was immobilized and only 44% of the activity was expressed (Table 1). The 3G3K PGA was completely immobilized (98%), but the expressed activity

Table 1. Immobilization of commercial wild-type (wt) and 3G3K PGAs on glyoxyl agarose and glyoxyl Sepabeads.

PGA	Conditions	Support	Loaded activity [IU/g of support][a]	Immobilized protein [%][b]	Activity [IU/g]	Yield [%][c]
wt	pH 10; 25 °C	Agarose	13	87	9.5	73
wt	pH 10; 25 °C	Agarose	80	95	46.0	58
wt	pH 9.5; 4 °C	Agarose	20	30	5.0	25
3G3K	pH 9.5; 4 °C	Agarose	10	51	3.0	30
3G3K	pH 9.5; 4 °C	Agarose	100	60	30.0	30
wt	pH 10; 25 °C	Sepabeads	43	83	19.0	44
wt	pH 9.5; 4 °C	Sepabeads	43	33	13.0	30
3G3K	pH 9.5; 4 °C	Sepabeads	20	98	1.0	5

[a] IU measured in the hydrolysis of PGK at 3% concentration, pH 8 and 37 °C (one unit liberates one μmol of phenylacetic acid per minute). [b] Determined by the Bradford assay.[36] [c] Immobilization yields were calculated as a percentage of activity expressed by the catalyst after immobilization.

was only 5%, significantly less than that of the commercial wild-type preparation.

These data prompted us to hypothesize that the loss of activity of 3G3K might be the consequence of the detrimental influence of the hydrophobic support. In fact, if we consider the stability of the 3G3K mutant in the immobilization conditions (pH 9.5, 4 °C),[32] this enzyme loses about 50% of its activity within 2 hours. When the mutant is immobilized on glyoxyl agarose, it maintains all the residual activity, as above explained, whereas when Sepabeads is used as the support, the activity of the immobilized biocatalyst dramatically decreases. We concluded that glyoxyl agarose is the best support for the tagged 3G3K PGA mutant.

The commercial wild-type enzyme showed an immobilization behaviour similar to that of the native wild-type PGA prepared as previously reported[32] (data not shown).

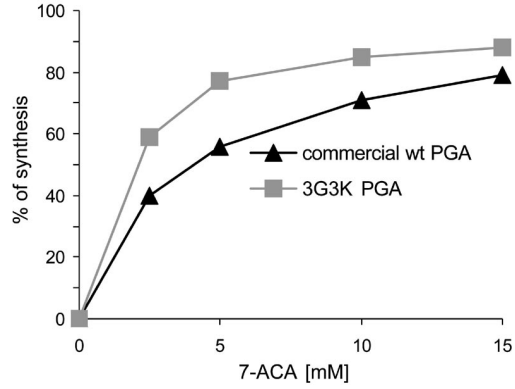


Figure 1. Acylation of 7-ACA at different concentrations with ester **1** catalyzed by commercial wild-type and 3G3K PGAs immobilized on glyoxyl agarose.

Determination of the vs/vh_1 Ratio in the Acylation of 7-ACA

The initial vs/vh_1 ratio was evaluated in the acylation of 7-ACA at different concentrations, with 5 mM ester **1** (Scheme 2), by comparing the commercial wild-type and the 3G3K PGA both immobilized on glyoxyl agarose. The vs/vh_1 value allows us to calculate the amount of ester utilized for the synthesis of the product **2a** at the beginning of the reaction (when the hydrolysis of the product is negligible) by using the following equation: $100 \times vs/(vs + vh_1)$. The vs/vh_1 ratio indeed can be considered as a descriptor of the percentage of synthesis. Figure 1 shows the percentage of synthesis versus the concentration of 7-ACA.

The wt acylase, immobilized by multipoint interactions (at 25 °C and pH 10), gave poorer performances (Figure 1) at all the tested concentrations of 7-ACA (particularly at low nucleus concentration) compared with the results achieved by using the 3G3K mutant (immobilized at 4 °C and pH 9.5).

In fact, with the immobilized 3G3K PGA, the percentage of synthesis was about 78% at a 5 mM concentration of 7-ACA, but was only 55% with the commercial wild-type PGA. In contrast, at a 15 mM concentration of 7-ACA, the two enzymes displayed similar behaviour: the percentage of synthesis was 90 and 80% for the mutant and the wt acylase, respectively (Figure 1). Moreover, by using the immobilized 3G3K on glyoxyl agarose we obtained the same results as those achieved with the non-immobilized enzyme at all the tested concentrations of 7-ACA. In contrast, with the commercial wt PGA, the percentage of synthesis decreased after immobilization (data not shown), according to results previously described.[32]

The degree of covalent interaction between the enzyme and the support during the immobilization is pH- and temperature-dependent. As it cannot be excluded that the performance of the enzyme might be affected by the immobilization conditions, such as, for example, conformational movement constraints due to multipoint attachment, the wt PGA was also immobilized at 4 °C and pH 9.5. The resultant biocatalyst was compared in the *N*-acylation of ester **1** with that obtained under the standard conditions (25 °C and pH 10; Table 2).

The enzyme, immobilized under different conditions, displayed a similar vs/vh_1 value in the *kcNa* of 7-ACA to give **2a**. This result indicates, as previously reported,[21] that the performance of the PGA immobilized on glyoxyl agarose is not influenced by the immobilization conditions and the degree of interaction between the enzyme and the support. Also, the vs/vh_1 value is not influenced by the amount of enzyme loaded onto the matrix. In fact, the same results were obtained with the biocatalyst prepared by loading 20

Table 2. The vs/vh_1 ratio obtained with free wt PGA and the 3G3K mutant and different immobilized preparations.

PGA	Loaded activity [IU/g of support][a]	Immobilization conditions[b]	vs/vh_1 (±sd)[c]
wt	none	–	2.1 (±0.40)
wt	13	pH 10; 25 °C	1.25 (±0.31)
wt	20	pH 9.5; 4 °C	1.35 (±0.07)
3G3K	none	–	3.2 (±0.17)
3G3K	10	pH 9.5; 4 °C	3.2 (±0.40)

[a] IU measured in the hydrolysis of PGK at 3% concentration, pH 8 and 37 °C (one unit liberates one μmol of phenylacetic acid per minute). [b] Immobilization on glyoxyl agarose. [c] Reaction conditions: 5 mM substrate, 4 °C, pH 6.5.

Table 3. Preparative synthesis of different cephalosporins catalyzed by commercial wild-type and 3G3K PGAs immobilized on glyoxyl agarose.[a]

PGA	Nucleus	Product	vs [IU/g]	% of maximum-conversion (±s.d.)
wt	7-ACA	**2a**	6	55 (±0.3)
3G3K	7-ACA	**2a**	18	63 (±0.8)
wt	7-TACA	**3a**	6	64 (±0.1)
3G3K	7-TACA	**3a**	17	70 (±1.0)
wt	7-SACA	**4a**	7	61 (±2.0)
3G3K	7-SACA	**4a**	13	67 (±0.8)

[a] Reaction conditions: 20 mM nucleus, 60 mM ester **1**, 4 °C and pH 6.5. PGA: 50 IU_{PGK} (corresponding to 1.1 g of commercial enzyme 46 IU/g); 30 IU_{PGK}. (corresponding to 1.0 g of 3G3K 30 IU/g).

or 200 IU of the wt PGA (Table 1 and data not shown). The commercial wild-type enzyme showed behaviour similar to that of the native wt PGA prepared as previously reported[32] (data not shown).

The 3G3K mutant is naturally characterized by a higher vs/vh_1 ratio (Table 2) than the wt enzyme. After immobilization at 4 °C and pH 9.5, the 3G3K mutant showed the same synthesis/hydrolysis ratio (S/H) as that obtained with the free enzyme (Table 2). However, the synthetic performances of wt PGA were reduced after immobilization.

Enzymatic Synthesis of Cephalosporins

The quantitative transformation of different β-lactam nuclei (**2**–**4**) into the corresponding cephalosporins was studied for mandelyl-7-ACA (**2a**), Cefamandole (**3a**) and Cefonicid (**4a**) (Scheme 2). The *N*-acylations were carried out by using a 20 mM concentration of the nucleus and a 3:1 molar excess of the ester **1** to enhance the reaction towards the complete conversion of the nuclei into the acylation products. The catalysts were the commercial wild-type and the 3G3K acylases, both immobilized on glyoxyl agarose, prepared by loading 80 (at 25 °C and pH 10) and 100 IU/g (at 4 °C and pH 9.5), respectively (see Table 1).

In all the tested reactions, the mutant PGA gave higher yields than the wt enzyme. The highest result (Table 3) was obtained in the synthesis of **3a** with a conversion of 70%. The greatest difference between the two enzymes was observed in the synthesis of **2a**, for which the yields were 63% with 3G3K and 55% with the commercial immobilized wt PGA, respectively.

Moreover, the mutant showed a much higher synthetic activity in all the reactions: in fact, 3G3K PGA gave a much higher vs value (IU/g of immobilized enzyme) than the commercial wt PGA (Table 3).

To further confirm the improved synthetic activity of the mutant, we also evaluated the relative rate of synthesis of each enzyme by considering the hydrolysis of the Penicillin G potassium salt (PGK) as the reference value. The relative rate of synthesis was calculated as the ratio between the rate of synthesis in each reaction and the rate of PGK hydrolysis (vs/vh_{PGK}), measured under the same conditions as those used for the acylation reactions (20 mM substrate concentration, pH 6.5 and 4 °C). Compared with the commercial wt PGA, 3G3K showed a significantly higher ratio in all the performed *N*-acylations (Figure 2).

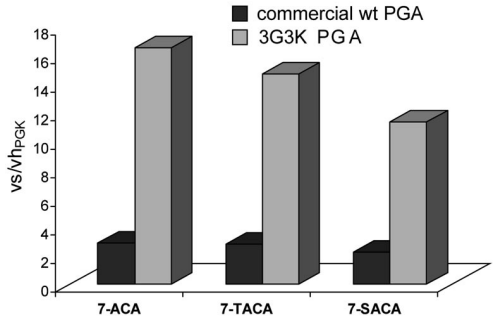


Figure 2. Calculated vs/vh_{PGK} ratios for different β-lactam nuclei.

The synthesis of Cefonicid (**4a**) was also performed at high concentrations (7-SACA: 50 mM; **1**: 150 mM). As shown in Figure 3, 3G3K PGA gave a higher rate of synthesis (≈16 IU/g) than the commercial wt immobilized preparation (≈7 IU/g) as well as a higher percentage of conversion. The maximum conversion (81%) was achieved by the mutant in only 90 min, whereas the native enzyme needed 180 min to reach the highest yield (76%). Note that, at high nucleus concentration (Figure 1), the difference in the vs/vh_1 ratio between the commercial and the mutant enzymes is minimal, but becomes important at low nucleus concentration (indeed, at the end of the synthesis). This is probably the most important effect that explains the slight improvement in the yield of antibiotic obtained in the preparative reaction catalyzed by the mutant compared with the wt acylase. Also, note that, after reaching the highest conversion, the product concentration rapidly decreases because hydrolysis of the product occurs (vh_2). This effect contributes to make less evident the difference between the two enzymes, with respect to expected yields, on the basis of different vs/vh_1 values.

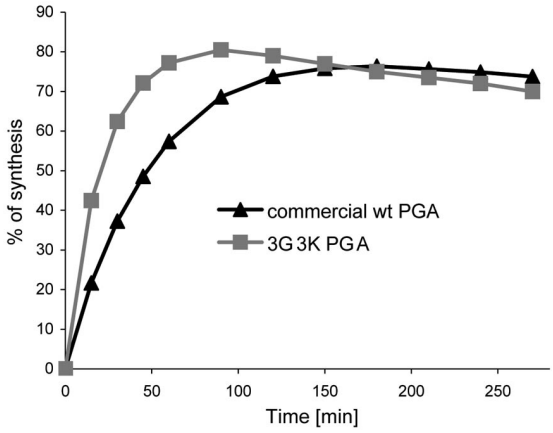


Figure 3. Synthesis of Cefonicid catalyzed by commercial wild-type and 3G3K PGAs immobilized on glyoxyl agarose.

Finally, it is important to point out that the immobilized mutant was stable under the experimental conditions. The 3G3K PGA, in fact, retained its activity almost completely after 24 h at 4 °C and pH 6.5 (results not shown).

Conclusions

As previously reported,[32] the 3G3K mutant, compared with the native enzyme, naturally shows an improved vs/vh_1 ratio, although the specific activities of these two acylases in the hydrolysis of PGK and ester **1** are very similar. This might be a consequence of a modification of the protein structure caused by the introduced mutations. The results described herein indicate that the 3G3K mutant of the PGA derived from *E. coli*, immobilized on glyoxyl agarose, is a more efficient catalyst than the wt enzyme for the preparative synthesis of different cephalosporins such as Cefamandole and Cefonicid. The differences between the 3G3K and the commercial wild-type PGA might be ascribed to the improved synthetic activity that characterizes the mutant. In fact, the 3G3K PGA not only displays a much higher vs/vh_1 ratio (even after immobilization), but also a much higher synthetic activity than the commercial wt enzyme. This suggests that the 3G3K mutant possesses an inherently higher specificity for transferase activity than for hydrolytic activity.

The differences between the wt and the mutant PGA become particularly evident after immobilization. Considering that immobilization on glyoxyl agarose mostly involves the surface enzyme amino groups (lysines) and that the 3G3K possesses a tag containing a sequence of alternating lysines far from the active site, we can hypothesize that this tag can somehow "drive" the immobilization process. Consequently, after immobilization, the β-lactam nucleus can freely access the active site of 3G3K, which maintains its inherent good synthetic properties, as reported for other mutants enriched with additional lysines.[30] In contrast, the wt enzyme is negatively affected by the immobilization.

These results demonstrate that, by inserting mutations in domains located far from the active site, it is possible to influence the catalytic properties of an enzyme and, most importantly, to preserve these properties after immobilization. Immobilization and site-directed mutagenesis can be exploited in a synergic way with the goal of designing more efficient catalysts.

The main drawback of the immobilized 3G3K PGA for potential large-scale applications (i.e., industrial use) is its poor stability in comparison with the wt enzyme. If the conditions of synthesis (low temperature and neutral pH) do not negatively affect the stability of the mutant, the use of co-solvents (methanol), as required in the synthesis of Cefonicid,[34] would not be compatible, unless optimization of the immobilization protocol and/or post-immobilization stabilization strategies are pursued.

Experimental Section

General: The extracts of PGA from *E. coli* were from Farmaopera (Opera, Milano, Italy); the protein concentration was 100 mg/mL with a specific activity towards PGK of 14 IU/mg. Cross-linked 6% agarose beads (Sepharose 6B-CL) were from Amersham Biosciences AB (Uppsala, Sweden). The β-lactam nuclei were donated by Farmabios s.r.l. (Gropello Cairoli, Pavia, Italy). Ester **1** was purchased from Sigma–Aldrich (Milano, Italy). All reagents and solvents were commercially available. HPLC analyses were performed with a BIO-TEK Kontron Instrument 422 equipped with a UV detector 535. The column used was a RP18 Interchrom 300×3.9 mm. During the enzymatic hydrolyses and syntheses the pH was kept constant by using an automatic 718 Stat Tritino titrator from Metrohm (Herisau, Switzerland).

Enzyme Over-Expression and Purification: The over-expression and purification of 3G3K PGA were performed by following the general procedure previously reported.[32] The protein concentration was 1.3 mg/mL with a specific activity towards PGK of 12.3 IU/mg.

Enzyme Activity: The PGA activity (μmol of hydrolysed Penicillin G potassium salt, PGK, $min^{-1} g^{-1}$ of immobilized enzyme) was measured by using a 3% solution of PGK at 37 °C and pH 8. The hydrolysis was followed by automatic titration with NaOH (100 mM). The activity was calculated from the consumption of NaOH.

Preparation of Glyoxyl Agarose and Immobilization: The preparation of glyoxyl agarose and immobilization were performed according to the previously reported general procedure.[35] Immobilization on glyoxyl Sepabeads was performed by following the procedure used for glyoxyl agarose. The protein concentration was determined by Bradford assay.[36]

Determination of vs and vh_1 in the Acylation of 7-ACA: The vs/vh_1 ratio of the PGA-catalyzed *N*-acylations with ester **1** was evaluated by measuring the initial rate of the synthesis (vs) of the acylation product and of the rate of ester hydrolysis (vh_1) to give (*R*)-mandelic acid at the beginning of the reaction (before 20% of ester was converted). The reactions were performed at pH 6.5 and 4 °C. According to the general procedure, the ester was dissolved in a solution of the β-lactam nucleus in 10 mM KH_2PO_4 buffer (20 mL). The immobilized PGA (10 IU) was added to the reaction at 4 °C under mechanical stirring (200 rpm). During the reaction the pH was kept constant by automatic titration. The reactions were monitored by HPLC analysis at 220 nm (eluent: 30% CH_3CN in 10 mM

phosphate buffer, pH 3.1) after removing the immobilized enzyme by sub vacuum (s.v.) filtration.

Cephalosporin Synthesis: The *N*-acylation of different nuclei was studied at pH 6.5 and 4 °C by using a molar excess of the acylating ester **1**. Following the general procedure, the desired amount of nucleus was dissolved in 10 mM phosphate buffer (20 mL) at pH 6.5. The ester was then added and the suspension stirred until complete solubilization. The immobilized preparation of PGA (50 IU) was added to the reaction at 4 °C under mechanical stirring (200 rpm). The reactions were monitored by HPLC analysis at 274 nm (eluent: 30% CH_3CN in 10 mM phosphate buffer pH 3.1 for **2a**; 20% CH_3CN in 10 mM phosphate buffer pH 3.2 for **3a** and **4a**) after removing the immobilized enzyme by s.v. filtration.

Determination of vh_{PGK}: The vh_{PGK} value was evaluated by measuring the rate of PGK (20 mM) hydrolysis to give phenylacetic acid. The reactions were performed at pH 6.5 and 4 °C. The immobilized PGA was added to the reaction under mechanical stirring (200 rpm). The hydrolysis was followed by automatic titration with NaOH (50 mM). The activity was calculated from the consumption of NaOH.

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