Pergamon

Bioorganic & Medicinal Chemistry Letters 11 (2001) 2429–2432

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Modulation of Penicillin Acylase Properties via Immobilization Techniques: *One-Pot* Chemoenzymatic Synthesis of Cephamandole from Cephalosporin C

Marco Terreni,[a,*] Giuseppe Pagani,[a] Daniela Ubiali,[a] Roberto Fernández-Lafuente,[b] Cesar Mateo[b] and José M. Guisán[b]

[a]*Pharmaceutical Biocatalysis Laboratories, Dipartimento di Chimica Farmaceutica, Università degli Studi di Pavia, via Taramelli 12, 27100 Pavia, Italy*

[b]*Laboratorio de Tecnologia Enzimatica, Instituto de Catalisis, C.S.I.C. Cantoblanco, 28049 Madrid, Spain*

Received 29 January 2001; revised 16 May 2001; accepted 22 June 2001

Abstract—The modulation of penicillin G acylase (PGA) properties via immobilization techniques has been performed studying the acylation of 7-aminocephalosporanic acid with *R*-mandelic acid methyl ester. PGA from *Escherichia coli*, immobilized onto agarose activated with glycidol (glyoxyl–agarose), has been used for the design of a novel *one-pot* synthesis of Cephamandole in aqueous medium and without isolation of intermediates, through three consecutive biotransformations catalyzed by D-amino acid oxidase, glutaryl acylase and PGA.

The use of enzymes is a powerful tool for the organic chemist. However, in order to take full advantage of the possibilities of these biocatalysts, mechanistic aspects must be considered, such as the conformational changes some enzymes can undergo during catalysis. For example, it has been hypothesized that penicillin G acylase (PGA) from *Escherichia coli* undergoes conformational modifications upon binding of the acyl donor substrate on the active center[1–3] and this has also been supported by crystallography analyses.[4] Consequently, it may be expected that any alteration of the conformation may induce significant changes of the enzyme properties. The use of immobilization protocols yielding enzyme derivatives with different rigidity or involving different areas of the protein may enable the modulation of the enzyme catalytic properties.

PGA from *E. coli* can be immobilized both on Eupergit C and on agarose gel activated with glycidol (glyoxyl–agarose).[5] Using Eupergit C, the immobilization of PGA proceeds via a two-step mechanism:[6–9] a preliminary hydrophobic absorption to the support is followed by a covalent attachment involving the nucleophilic groups of the enzyme (Lys, Cys, Tyr) and the epoxy groups of the support. The enzyme is mostly immobilized, indeed, through its hydrophobic areas. On the contrary, using glyoxyl–agarose,[10] the enzyme is immobilized through the areas bearing the highest density of Lys residues.[11] Consequently, the immobilization on Eupergit C and glyoxyl–agarose can induce a different orientation of the protein. Furthermore, the immobilization on glyoxyl–agarose may yield enzyme derivatives with different rigidities and stabilities depending on the activation degree of the support: a limited (not stabilized) or a multipoint (stabilized) linkage can be obtained using a low or a high activated support, respectively.[5]

Using PGA from *E. coli* a new chemoenzymatic synthesis of Cefazolin from Cephalosporin C has been proposed.[12] This approach, performed in fully aqueous medium and without intermediates purification, involves three consecutive biotransformations catalyzed by D-amino acid oxidase (DAAO), glutaryl acylase (GA) and PGA, respectively.

In this process, the acylation of 7-ACA with tetrazol-1-yl acetic acid methyl ester (TZAM) catalyzed by immobilized PGA was the key step to be optimized in order to obtain the final product in high yield and concentration.

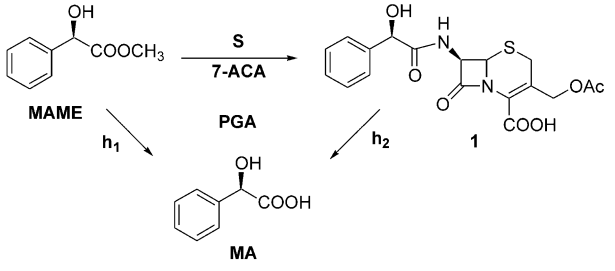
*Corresponding author. Tel.: +39-0382-507625; fax: +39-0382-507589; e-mail: markt@pbl.unipv.it

0960-894X/01/$ - see front matter
PII: S0960-894X(01)00463-2

When mandelic acid methyl ester (MAME) was used instead of TZAM, the acylation of 7-ACA was unsuccessful.[12] For this reason, we decided to study and to try to optimize this enzymatic reaction via biocatalyst and engineering reaction. We prepared different derivatives of PGA isolated from *E. coli* (Recordati; Opera, Italy). This enzyme was immobilized on agarose according to the procedure previously reported.[5] In order to evaluate the influence of the rigidity of the enzyme structure on the catalytic properties, PGA was immobilized on agarose activated with 15 and 75 μmol of glyoxyl groups per mL of support, affording a limited and a multipoint covalent derivative, respectively. The enzyme was also immobilized on Eupergit C (Rhon; Darmstadt, Germany) by interaction at pH 8 in presence of 1 M phosphate buffer. The properties of the derivatives prepared were tested in the enzymatic acylation of 7-ACA with MAME, to obtain 7-[(1-hydroxy-1-phenyl)-acetamido]-3-acetoxymethyl-Δ^3-cephem-4-carboxilic acid **1** (Scheme 1), an intermediate for the preparation of Cephamandole and other 3′-functionalized cephalosporins. The optimization of the reaction conditions and the assembly of this enzymatic step with the DAAO/GA catalyzed cleavage of Cephalosporin C were also considered in order to develop a multi-enzymatic *one-pot* synthesis of Cephamandole (see Scheme 1).

In the kinetically controlled acylation catalyzed by PGA, the yields depend on the balance of three different reactions catalyzed by the same enzyme (Scheme 1):[12] the synthesis of the β-lactamic compound (S), the undesired hydrolyses of the activated acyl donor (h_1) and of the product (h_2). The yield depends on the ratio between the rate of synthesis (Vs) and the rate of the two hydrolyses (Vh_1 and Vh_2): to obtain good yields the ratios Vs/Vh_1 and Vs/Vh_2 must be as high as possible.

For the study of the catalytic properties, the ratio between the esterase and amidase activities (Ve/Va) was considered as a parameter strictly related to Vs/Vh_2. The enzyme showing the highest Ve/Va ratio may be expected to present the highest synthetase/amidase activity ratio.[12] This ratio was evaluated measuring, separately, the hydrolytic activity against MAME (Ve) and against Cephamandole (Va). Cephamandole was selected as reference substrate in order to evaluate the capacity of the catalyst to hydrolyze the amidic bond of cephalosporin compounds bearing *R*-mandelic acid as side chain. The esterasic activity is, instead, a measure of the capacity of the catalyst to accept the acylating agents in the active center, the first step of the synthetic process.[12]



Scheme 1.

The Vs/Vh_1 ratio was instead evaluated monitoring directly the formation of the acylation product **1** and mandelic acid (MA) at the beginning of the acylation reaction (less than 20% of ester consumed) between 7-ACA and MAME. The acylations were carried out using a high excess of the β-lactam nucleus in order to consider constant its concentration, ensuring a suitable saturation degree of the enzyme active sites.

The results obtained with the two agarose derivatives (limited and multipoint linkage) were very similar concerning both the Ve/Va and the Vs/Vh_1 ratios, suggesting that the rigidity of the immobilized enzyme does not influence the catalytic properties (Table 1).

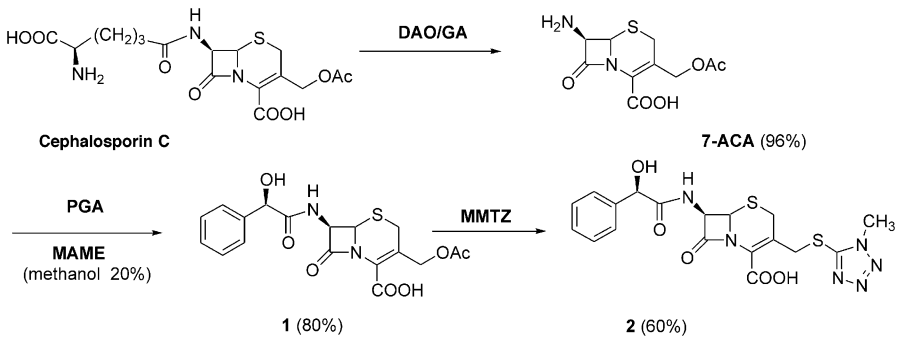
From the comparison of the PGA derivatives immobilized on agarose and Eupergit C, significant differences of the Ve/Va ratio were not observed. In fact, all derivatives showed a low value due to a high hydrolytic activity against the amide (Cephamandole). The effects of the immobilization were more significant for the Vs/Vh_1 ratio and the most interesting values were obtained with the agarose derivatives (Table 1).

The derivatives were also studied in extreme experimental conditions (high ionic strength or presence of water mixable solvents). Comparing the enzyme immobilized on Eupergit C and agarose by multipoint covalent interaction, the effects of the different tested conditions were very different; agarose–multipoint derivative always showed the best catalytic properties. For this derivative, high concentrations of phosphate buffer increased the Ve/Va ratio, whereas methanol decreased the Vs/Vh_1 ratio. High ionic strength decreased the amidase activity against Cephamandole. In fact, although the esterasic activity was unaffected, when phosphate buffer concentration was 1 M, the Ve/Va ratio was 7-fold higher than the value obtained with low ionic strength media. The Vs/Vh_1 ratio was unaffected by high ionic strength, but increased (from 8 to up to 20) in the presence of methanol (Table 1). For the enzyme immobilized on Eupergit C, high concentrations of phosphate buffer allowed a very high activity to be obtained (Ve around 50 μmol/min×g of enzyme derivative), but the Ve/Va ratio slightly decreased. In the presence of methanol, the value of Vs/Vh_1 ratio was only 7 (Table 1).

Table 1. Influence of the enzyme derivative and of the reaction conditions on the catalytic properties of PGA (4 °C; pH 6.5)

Support	Phosphate buffer	Solvent (20%)	Ve[a]	Ve/Va	Vs/Vh_1
Eupergit C	10 mM	—	15	0.67	2.4
Agarose–limited linkage	10 mM	—	12	1.09	7.5
Agarose–multipoint	10 mM	—	25	0.94	8.2
Agarose–multipoint	500 mM	—	31	4.70	8.0
Agarose–multipoint	1 M	—	29	8.43	9.4
Agarose–multipoint	10 mM	Methanol	4.7	0.61	>20
Eupergit C	1 M	—	44	0.51	1.8
Eupergit C	10 mM	Methanol	3.3	0.75	7.1

[a]Activity expressed in U/g (μmol of substrate hydrolyzed/min×g of enzyme derivative).



Scheme 2.

These results show that the reaction conditions may be very important for the optimization of a kinetically controlled reaction catalyzed by PGA, but note that the immobilization of the enzyme can be crucial for developing efficient catalysts. The differences of the derivatives on Eupergit C and agarose may be ascribed to a change in the orientation of the protein, depending on the different immobilization mechanism. However, the effect of the nature of the support cannot be underestimated.

In order to obtain the quantitative transformation of the β-lactamic nucleus into compound **1**, the acylation of 50 mM 7-ACA was performed using a 3:1 molar excess of MAME and the agarose–multipoint derivative as catalyst. In standard conditions (10–25 mM phosphate buffer, pH 6.5, 4 °C) the yield (Fig. 1, triangles) was low (69%). The simultaneous use of methanol and a high ionic strength (1 M phosphate buffer) improved the yield up to 80% (Fig. 1, squares), in agreement with the results obtained studying the catalytic properties of the enzyme in the same reaction conditions.

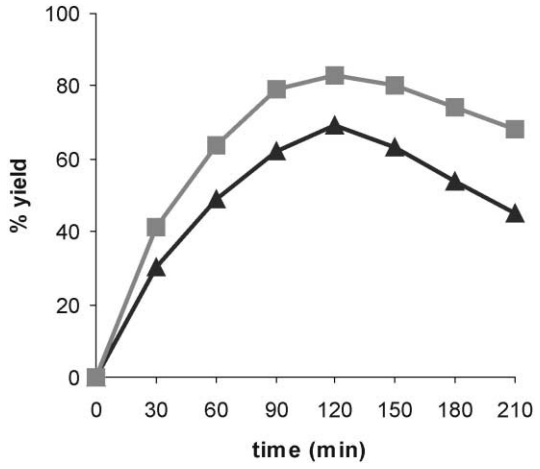


Figure 1. Influence of the reaction conditions on the acylation of 7-ACA (10–25 mM phosphate buffer, -▲-; 1 M phosphate buffer and 20% methanol, -■-).

To develop a multienzymatic *one-pot* synthesis of Cephamandole **2** in good yield, the reaction catalyzed by PGA was assembled with the DAAO/GA catalyzed deacylation of Cephalosporin C (Scheme 2). The first step was performed in 1 M phosphate buffer (pH 8), at rt and under O_2 atmosphere. The reaction afforded 7-ACA in good yield (96%) and high final concentration (60 mM) in only 3 h. The acylation catalyzed by PGA on agarose was then performed on the crude solution of 7-ACA (pH 6.5, 4 °C) after filtration of the immobilized DAAO and GA, using MAME (150 mM, 3:1 molar ratio) in 20% methanol (80% yield). After filtration of the PGA derivative, the final displacement of the 3-acetoxyl group with 1-methyl-5-mercaptotetrazole (MMTZ) was performed by heating directly the aqueous solution of **1** to 65 °C (60% yield of **2**).

The results obtained in this work for the acylation of 7-ACA with MAME demonstrate that the immobilization can considerably modulate the enzymatic catalytic properties. The assembly of this enzymatic step with the DAAO/GA catalyzed cleavage of Cephalosporin C, allowed a chemo-multienzymatic *one-pot* synthesis of Cephamandole to be performed in aqueous medium, giving the desired product with acceptable yield and good concentration, without any intermediates purification. The suggested approach can be considered generally useful for the synthesis of 3′-functionalized cephalosporins bearing *R*-mandelic acid as side chain in position 7. Besides, several practical advantages emerge in comparison with the conventional chemical process; particularly, the use of toxic reagents and chlorinated solvents is avoided, and the substrate specificity and selectivity of the enzymes make unnecessary the protection–deprotection of reactive groups, reducing chemical and purification steps.

Acknowledgements

This research was partially supported by Recordati S.A. The authors would like to thank Rafael Armisen (Hispanagar S.A.) for his generous gift of agarose gels and Recordati S.A. for providing the enzymes.

References and Notes

1. Martin, J.; Manchego, J. M.; Arche, R. *Biochem. J.* **1993**, *291*, 907.
2. Siewiinsky, M.; Kuropatwa, M. *Hoppe-Seyler's Z. Physiol. Chem.* **1984**, *635*, 829.

3. Fernandez-Lafuente, R.; Rosell, C. M.; Guisan, J. M. *Enzyme Microb. Technol.* **1998**, *22*, 583.
4. Done, S. H.; Branningan, J. A.; Moody, P. C. E.; Hubbard, R. E. *J. Mol. Biol.* **1998**, *284*, 463.
5. Álvaro, G.; Fernández-Lafuente, R.; Blanco, R. M.; Guisán, J. M. *Appl. Biochem. Biotechnol.* **1990**, *26*, 181.
6. Melander, W.; Corradini, D.; Hoorvath, C. S. *J. Chromat.* **1984**, *317*, 67.
7. Smalla, K.; Turkova, J.; Coupek, J.; Herman, P. *Biotechnol. Appl. Biochem.* **1988**, *10*, 21.
8. Wheatley, J. B.; Schmidt, D. E. *J. Chromat.* **1993**, *644*, 11.
9. Wheatley, J. B.; Schmidt, D. E. *J. Chromat. A* **1999**, *849*, 1.
10. Guisan, J. M. *Enzyme Microb. Technol.* **1988**, *10*, 375.
11. Guisán, J. M.; Penzol, G.; Armisen, P.; Bastida, A.; Blanco, R. M.; Fernández-Lafuente, R., García-Junceda, E. In *Methods Biotechnol.*; Bickerstaff, G., Ed.; Humana: Totowa, 1997; Vol. 1, p 261.
12. Hernández-Justiz, O.; Fernández-Lafuente, R.; Guisán, J. M.; Negri, P.; Pagani, G.; Pregnolato, M.; Terreni, M. *J. Org. Chem.* **1997**, *62*, 9099.