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# On the substrate preference of glutaryl acylases

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ABSTRACT

The substrate preferences of three acylases – two wild-type enzymes and an evolved variant obtained by directed evolution – which are prototypical enzymes for glutaryl-7-ACA acylase and cephalosporin C acylase subfamilies, have been investigated. A preliminary screening of enzymes' performances on a large set of substrates has been carried out by a colorimetric assay performed in 96-well plates and by a pH-Stat monitoring the hydrolytic activities. Subsequently, kinetic data for selected substrates have been determined, thus elucidating the substrate preference of members of glutaryl-7-ACA acylase vs. cephalosporin C acylase subfamilies. These achievements pave the way to the ability of choosing the best enzyme for the hydrolysis of different compounds of industrial importance.



## 1. Introduction

The use of enzymes as industrial biocatalysts offers significant advantages when compared to chemical processes: enzymes show high activity under mild conditions – making them "green" catalysts (from an environmental and energetic point of view) – and possess high specificity. Accordingly, enzymes can be quite often employed on complex mixtures (thus avoiding the use of pure substrates) and can perform stereo- and regioselective reactions [1–5]. However, strict/narrow specificity can affect the recognition of structurally similar or related compounds, thus limiting the industrial exploitation of these enzymes. Therefore, new biotechnological applications of biocatalysts may be largely dependent on a broad substrate acceptance, nowadays also called substrate promiscuity [6,7].

Glutaryl acylase (EC 3.5.1.93), a member of the N-terminal hydrolases class of hydrolytic enzymes, is a well known industrial biocatalyst showing a wide substrate specificity. Glutaryl acylases have been identified in a variety of microorganisms [8] and two alternative classification systems have been proposed: (i) the members are grouped based on their affinity to cephalosporin C (CephC) [9]: enzymes with a detectable activity on CephC are named "cephalosporin C acylases" (CA), the others belong to the "glutaryl-7-ACA acylases" (GA); (ii) in the alternative system, glutaryl acylases are grouped into five classes based on their sequence, molecular mass and enzymatic properties [10]: members of each subclass are very similar according to substrate specificity and sequence conservation (i.e. they share more than 90% of nucleic or amino acidic sequence identity). These enzymes show activity toward CephC that ranges from 0% to 4% relative to glutaryl-7-aminocephalosporanic acid (Gl-7-ACA) [11].

The most important application of glutaryl acylases is the two-step process comprising the conversion of CephC into Gl-7-ACA using the enzyme D-amino acid oxidase (EC 1.4.3.3) [12–15], and its subsequent hydrolysis to 7-aminocephalosporanic acid (7-ACA) by a glutaryl acylase [8,16,17]. Indeed, GA enzymatic activity can be employed to hydrolyze various glutaryl esters [8,17,18] or for the opposite synthetic reaction. It has been shown that these acylases require an amide carrying a carboxylate side chain (Gl-7-ACA is the best substrate but derivatives having succinyl or adipoyl groups can be efficiently hydrolyzed as well); furthermore, the amine

*Abbreviations:* 7-ACA, 7-aminocephalosporanic acid; 7-ADCA, 7-aminodesacetoxycephalosporanic acid; CA, cephalosporin C acylase; CephC, cephalosporin C; pDMAB, p-4-dimethylaminobenzaldehyde; GA, glutaryl acylase; Gl-7-ACA, glutaryl-7-aminocephalosporanic acid; Gl-7-ADCA, glutaryl-7-aminodesacetoxycephalosporanic; Gl-7-ZACA, glutaryl-7-Z-aminocephalosporanic acid; Gl-3Cl-7-ADCA, glutaryl-3-chloro-7-aminodesacetoxycephalosporanic acid; Glut-L-Ala, glutaryl-L-alanine methylester; Glut-Gly, glutaryl-glycine methylester; Glut-D-phenylGly, glutaryl-D-phenylglycine methylester; Glut-L-Phe, glutaryl-L-phenylalanine methylester; Glut-L-phenylGly, glutaryl-L-phenylglycine methylester.

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substituent can also be quite different from a β-lactam skeleton [18]. Using an array of racemic glutarate derivatives, enantioselective amidase and esterase activities have been also observed [8,19,20].

In this work we compare the substrate preference of a GA widely used in industry (glutaryl acylase commercialized by Recordati S.p.A., GAR) [16,18,19] with an evolved CA obtained by a combined approach based on error-prone PCR mutagenesis, site-saturation mutagenesis and site-directed mutagenesis driven by a molecular modelling analysis (VAC, both the wild-type and H296S-H309S variant) [21].

## 2. Experimental

### 2.1. Enzymes

Commercial glutaryl acylase GAR was a gift from Recordati S.p.A. (Opera, MI, Italy); its specific activity on Gl-7-ACA is 2.3 U/mg protein [20]. Recombinant VAC proteins (wild-type and H296S-H309S VACs) were produced as stated in [22,23]. From 1 L of fermentation broth, 55 and 77 mg of wild-type and H296S-H309S VACs were produced with a specific activity of 20.2 and 2.4 U/mg protein on Gl-7-ACA as a substrate, respectively. The final enzyme preparations were equilibrated in 20 mM potassium phosphate buffer, pH 8.0. The amount of protein was estimated by the absorbance at 280 nm using the molar extinction coefficient of 110 $mM^{-1}$ $cm^{-1}$.

### 2.2. Substrate synthesis

Cephalosporanic amides were prepared by reaction with a suitable anhydride as described in our previous paper [18]. Briefly, the substrate – either 7-ACA, 7-aminodesacetoxycephalosporanic acid (7-ADCA), 7-Z-aminocephalosporanic acid or 3 chloro-7-aminodesacetoxycephalosporanic acid, 5 mmol – was dissolved in 20 mL of 1 M $NaHCO_3$, while the anhydrides (1 equiv.) were diluted in 5 mL acetone. The two solutions were mixed and let to react for at least 3 h (TLC: *n*-BuOH:AcOH:$H_2O$ = 6:2:2). Acetone was evaporated, the water solution was acidified to pH 1.5 with 1 M HCl and extracted three times with 100 mL AcOEt. The organic layer was evaporated and the solid residue was washed on a Buchner funnel with 20 mL AcOEt and dried. Amino acidic derivatives were prepared by a similar procedure, but dissolving both the substrate (5 mmol) and the anhydride (1 equiv.) in anhydrous dioxane (30 and 5 mL, respectively), and maintaining the reaction under stirring at 50 °C for at least 24 h. After evaporation of dioxane, the product was redissolved in 20 mL of water and recovered as described previously.

### 2.3. Enzymatic activity and kinetic measurements

The standard activity assay was based on the hydrolysis of Gl-7-ACA to 7-ACA and the subsequent formation of a yellow Shiff's base (with a maximum of absorbance at 415 nm, $\varepsilon = 0.635\ M^{-1}\ cm^{-1}$) by the reaction of *p*-4-dimethylaminobenzaldehyde (pDMAB) and the primary amino group of 7-ACA [21,24]. One unit of acylase is defined as the amount of enzyme that converts 1 μmol of substrate per minute under the assay conditions. In details: 0.1 mL of enzyme was mixed with 0.1 mL of 1% (w/v) Gl-7-ACA at pH 8.0, and incubated for 10 min at 25 °C. The reaction was stopped by adding 0.6 mL of 20% acetic acid, and then 0.133 mL of 0.5% (w/v) pDMAB (dissolved in methanol) was added and the mixture was incubated for further 10 min at 25 °C, before measuring the absorbance at 415 nm. The kinetic parameters were determined similarly using a fixed amount of enzyme and different substrate concentrations (0–150 mM): activity vs. substrate concentration data were analyzed according to the classical Michaelis–Menten equation or modified to account for a substrate inhibition effect [25,26].

### 2.4. Screening for acylase activity on different substrates

The acylase activity on different compounds was assayed by a semi-quantitative colorimetric screening procedure based on the reaction of pDMAB with the free $NH_2$-group produced starting from different substrates. The extinction coefficient of each Schiff's base is different depending on the substrate used, this affecting both the precision and sensitivity of the measurement. In details, 50 μL of enzyme and 50 μL of substrate (5 or 50 mM final concentrations) were transferred to wells of a 96-well plate and incubated at 25 °C for 10 min. The reaction was stopped by adding 100 μL of stop solution (30% acetic acid); then 50 μL of 0.35% (w/v) pDMAB in methanol was added. After 10 min of incubation at 25 °C, the absorbance value at 405 nm was measured by a microtiter plate reader (Sunrise, TECAN) and compared with the value obtained with Gl-7-ACA as reference. In addition to the substrates reported in Table 1, the following compounds have been tested: glutaryl-D-alanine, -phenylalanine, -phenylglycine; glutaryl-L-alanine, -phenylalanine, -phenylglycine; methylesters of glutaryl-D-alanine, -phenylalanine, -phenylglycine, -leucine, -tryptophan, -alanine; methylesters of glutaryl-L-alanine, -phenylalanine, -phenylglycine, -leucine, -tryptophan, -alanine and glutaryl-glycine methylester; glutaryl *p*-nitrobenzyl amide; glutaryl 1-phenylethyl amide; glutaryl benzyl amide; glutaryl cyclobutyl amide; glutaryl cyclopentyl amide; glutaryl cycloesyl amide; succinyl benzyl amide.

### 2.5. Hydrolytic reactions (pH-Stat)

Hydrolytic reactions were monitored at 20 °C using a 718 STAT Titrino automatic titrator (Metrohm Ltd.). In a total volume of 10 mL, 50 mM substrate dissolved in $H_2O$ and a suitable amount of GAs were stirred while maintaining the pH at a constant value of 8.0 by adding 0.1 M NaOH. The rates of hydrolysis were calculated from the amount of NaOH solution added in the time unit. Relative activity on various substrates was compared with that observed for Gl-7-ACA, fixed as 100%.

## 3. Results

### 3.1. Analysis of substrate preference

A preliminary screening of the activity of wild-type and H296S-H309S VAC enzymes on a number of compounds was carried out by the pDMAB-based colorimetric assay using a 96-well plate. For these measurements two amounts of enzyme (0.75 and 7.5 μg for wild-type, 19 and 190 μg for H296S-H309S VAC) and two substrate concentrations (5 and 50 mM) were employed. At 5 mM substrate concentration, both VAC enzymes showed a negligible activity on amino acid derivatives of glutaric acid, as well as on the corresponding methyl esters or on glutaryl amides of cyclic amines (not shown). On the contrary, an increase in absorbance at 405 nm was observed with most of the tested cephalosporanic derivatives: the relative activity values – vs. Gl-7-ACA as a reference substrate, fixed as 100% – are reported in Table 1. At 50 mM substrate concentration a similar pattern was observed, the main exception being a substantial increase in activity on pentanoyl-7-ACA and glutaryl-3-chloro-7-aminodesacetoxycephalosporanic acid (Gl-3Cl-7-ADCA) for wild-type VAC and on pentanoyl-7-ACA and CephC for the H296S-H309S VAC variant. The low activity determined at 5 mM CephC with the latter enzyme (≈15% as compared to Gl-7-ACA) is mainly related to the comparatively high $K_m$ value for this substrate, see below and [21]. For some of the compounds tested, the activity values determined at 50 mM substrate concentration were

**Table 1**
Relative activity on different substrates of wild-type and H296S-H309S variant of VAC.

| Substrate | Wild-type VAC | | H296S-H309S VAC | |
|---|---|---|---|---|
| | 5 mM substrate | 50 mM substrate | 5 mM substrate | 50 mM substrate |
| Gl-7-ACA | 100 | 100 | 100 | 100 |
| Gl-7-ADCA | 71.5 ± 4.2 | 76.7 ± 6.2 | 70.0 ± 6.3 | 98.3 ± 4.6 |
| Succinyl-7-ACA | 25.8 ± 6.5 | 25.7 ± 1.0 | 29.4 ± 5.4 | 5.9 ± 1.1 |
| Adipyl-7-ADCA | 57.4 ± 1.4 | 62.8 ± 1.0 | 100.4 ± 4.3 | 104.6 ± 4.0 |
| Pentanoyl-7-ACA | ≈2.4 | 20.6 ± 1.6 | ≈4.1 | 57.2 ± 6.7 |
| Butanoyl-7-ADCA | b.d. | b.d. | ≈4.2 | 6.1 ± 2.0 |
| Pentanoyl-7-ADCA | b.d. | b.d. | ≈0.5 | 5.1 ± 1.8 |
| Gl-3Cl-7-ADCA | 81.9 ± 2.5 | 180.1 ± 7.0 | 79.9 ± 9.0 | 97.6 ± 3.0 |
| Gl-7-ZACA | 9.9 ± 0.1 | b.d. | 8.6 ± 3.2 | ≈2.4 |
| CephC | b.d. | 6.0 ± 1.0 | 14.8 ± 4.5 | 73.0 ± 4.3 |

The reported values are the average of results gathered using 0.75 and 7.5 μg of wild-type VAC or 19 and 190 μg of H296S-H309S VAC. b.d., below detection.

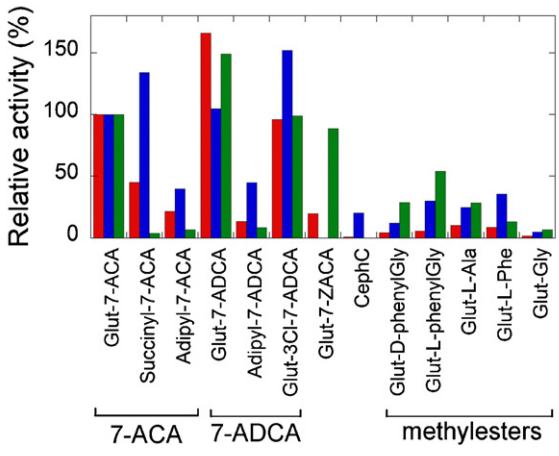


**Fig. 1.** Comparison of hydrolytic activity on different compounds of wild-type VAC (red), H296S-H309S variant VAC (blue) and GAR (green) as determined using the pH-Stat assay, at 20 °C and 50 mM substrate concentration. The values are reported as percentage with respect to the value determined on Gl-7-ACA, fixed as 100%. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

appreciably lower than at 5 mM (see below and Table 1). The data for GAR with the same substrates are available in literature [18] and were confirmed by our experiments (see below). Differently from the VAC enzymes, GAR showed a substantial activity also toward methyl esters of N-glutaryl-L-phenylglycine, -L-leucine, -L-alanine and L-phenylalanine (from 59.3 to 15.7% of the value determined for Gl-7-ACA).

Because the colorimetric assay based on pDMAB was strongly dependent on the substrate used (see Section 2), the activity was also assessed monitoring the hydrolysis by means of a pH-Stat at 50 mM substrate concentration. This assay, whose response does not depend on the coupling reaction with pDMAB and the different extinction coefficients of the resulting Shiff's bases, largely confirmed the previous results (Fig. 1). A very low activity of VAC enzymes on glutaryl-7-Z-aminocephalosporanic acid (Gl-7-ZACA, having a large substituent in C3), of GAR on succinyl-7-ACA, and a comparatively higher activity of GAR on amino acid derivatives of glutaric acid – the only exception being glutaryl-L-phenylalanine methylester which was more efficiently hydrolyzed by H296S-H309S VAC – was apparent.

### 3.2. Kinetic properties on cephalosporin derivatives

The evaluation of the kinetic parameters of GAR and of wild-type and H296S-H309S VACs on the cephalosporin derivatives identified by the previously described screening analysis was carried out spectrophotometrically on a 1 mL assay mixture containing increasing substrate concentrations.

#### 3.2.1. Glutaryl-7-ACA

As shown in Fig. 2A, the highest activity on Gl-7-ACA (and kinetic efficiency, expressed by the $V_{max}/K_m$ ratio) was observed for wild-type VAC, while both H296S-H309S VAC and GAR showed a similar behavior and a lower activity. The corresponding kinetic parameters are reported in Table 2. Noteworthy, a substrate inhibition effect was apparent for wild-type VAC at substrate concentrations >6 mM ($K_i \approx 20$ mM). Anyway, at 100 mM Gl-7-ACA – a concentration resembling conditions used for industrial bioconversions [12,13] – the activity was 2-fold higher with the latter enzyme than with either H296S-H309S VAC or GAR.

#### 3.2.2. Glutaryl-7-ADCA

Concerning the VAC acylases, the kinetic parameters of the wild-type enzyme on glutaryl-7-ADCA (Gl-7-ADCA) resembled those obtained with Gl-7-ACA – only the $K_i$ for the substrate inhibition was 3-fold higher (Fig. 2B) – and a 2-fold higher $K_m$ was apparent for the H296S-H309S variant (Table 2). Most significant changes were evident for GAR: a 2-fold decrease in $K_m$ value and a ≈3-fold increase in $V_{max}$ was observed for the Gl-7-ADCA when compared to the corresponding ACA derivative, thus yielding a 7-fold increase in kinetic efficiency (Table 2). At high substrate concentration (≥100 mM) GAR seemed as suitable as wild-type VAC for bioconversion of Gl-7-ADCA.

#### 3.2.3. Succinyl-7-ACA

The kinetic properties of the acylases under investigation were slightly affected by shortening the side chain at C7 of ACA moiety, as using succinyl-7-ACA as substrate: the most significant change being a 2-fold increase in $K_m$ for wild-type VAC (Table 2). Interestingly, and because of the substrate inhibition effect on wild-type VAC, at substrate concentrations ≥80 mM the H296S-H309S variant VAC showed an activity similar to the wild-type counterpart (Fig. 2C).

#### 3.2.4. Adipyl(/pentanoyl)-7-ACA and adipyl-7-ADCA

Significant changes were observed when a compound with longer side chain in C7 as compared to the reference substrate Gl-7-ACA was used. A major decrease in the $V_{max}$ value was apparent for all the enzymes tested (up to 12-fold decrease for GAR with adipyl-7-ACA) coupled to a 5-fold increase in $K_m$ for the wild-type VAC (Table 2 and Fig. 2E). A similar decrease in maximal activity was also apparent with adipyl-7-ADCA as a substrate vs. glutaryl-7-ADCA for wild-type VAC and GAR, a change not observed for the variant VAC. In fact, and because of the increase in $V_{max}$ value with the adipyl-7-ADCA vs. Gl-7-ADCA (Table 2), the highest activity at substrate

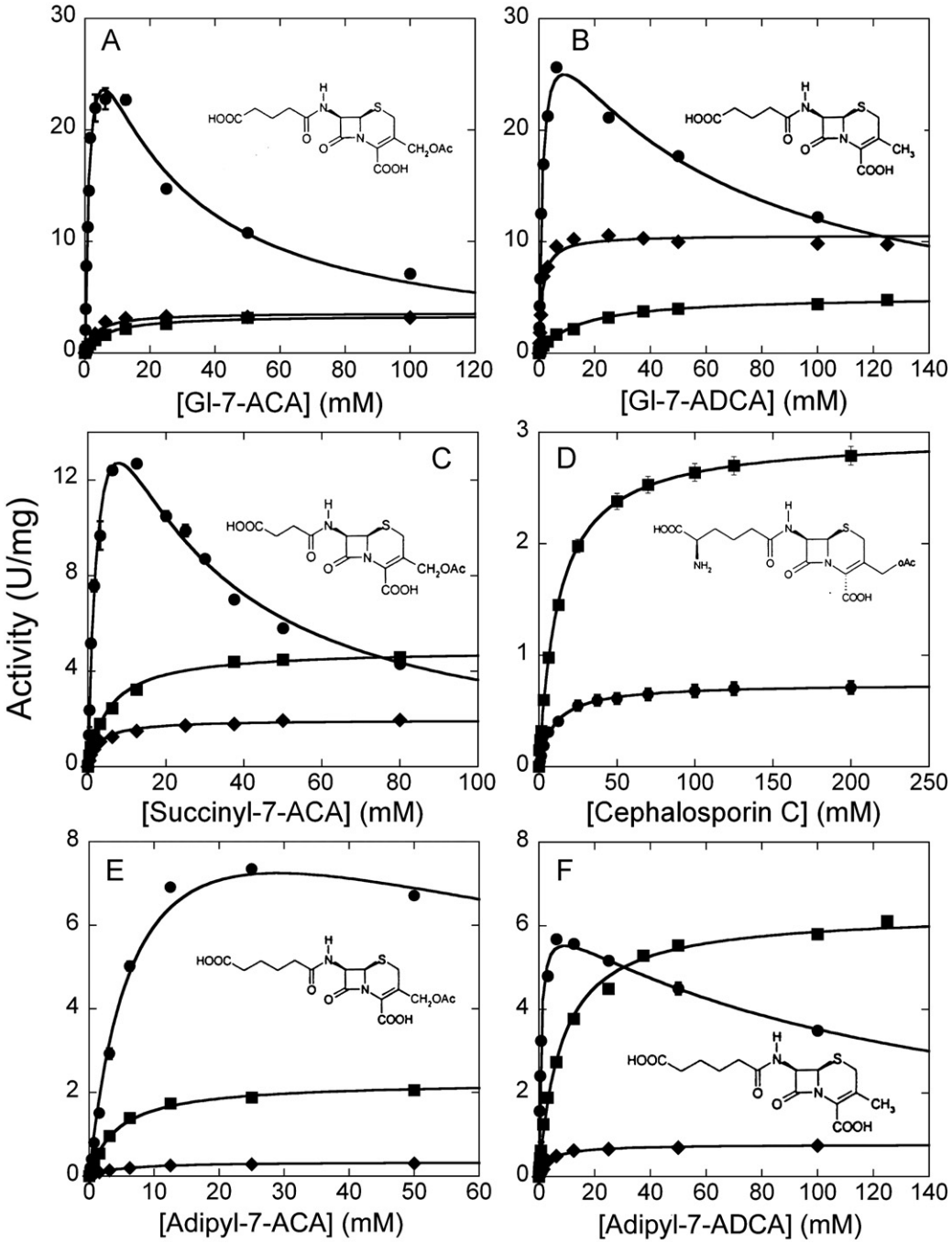


**Fig. 2.** Dependence of the activity values for the reaction of wild-type VAC (●), H296S-H306S VAC (■), and GAR (♦) from the concentration of different cephalosporin derivatives differing at C7. The data points were obtained as described in the text (average of at least 3 single experiments) and have been determined using different amounts of enzyme (wild-type VAC: 3.8–32 μg/assay; H296S-H306S VAC: 57–445 μg/assay; GAR: 50–700 μg/assay). Panel (A) Gl-7-ACA; (B) Gl-7-ADCA; (C) succinyl-7-ACA; (D) CephC; (E) adipyl-7-ACA; (F) adipyl-7-ADCA. The curves are from fits based on the classical Michaelis–Menten equation or its modification to account for a substrate inhibition effect [25,26].

concentration ≥40 mM was observed with the VAC double variant (Fig. 2F).

The lack of the carboxylate in the side chain at C7, as in pentanoyl-7-ACA, resulted in a full loss of enzymatic activity for GAR (no activity was observed up to 1 mg of enzyme/assay) and in a ≈20-fold decrease in $V_{max}$ for the VAC enzymes (Table 2).

### 3.2.5. Cephalosporin C

The kinetic parameters on the most valuable industrial substrate CephC, currently used as starting compound to produce semisynthetic cephalosporins [12–15], were determined for the two VAC variants only, since no activity was observed for the GAR enzyme up to 1 mg of enzyme/assay. The kinetic efficiency was ≈3-fold higher for H296S-H309S variant as compared to the wild-type VAC, due to a higher $V_{max}$ value (see Fig. 2D and Table 2).

### 3.2.6. Glutaryl derivates with C3 modifications

For all the enzymes tested, similar kinetic parameters were obtained with the Gl-3-Cl-7-ADCA as compared to Gl-7-ADCA as reference (Table 2). The kinetic efficiency of wild-type VAC was ≈40-fold and ≈6-fold higher than that of H296S-H309S VAC and GAR, respectively, mainly arising from the higher affinity for the

**Table 2**
Kinetic parameters of wild-type and H296S-H309S variant of VAC and of GAR on different substrates (see Figs. 2 and 3).

| Substrate | | Wild-type VAC | H296S-H309S VAC | GAR |
|---|---|---|---|---|
| *C7 derivates of 7-ACA and 7-ADCA* | | | | |
| Gl-7-ACA | $V_{max}$ (U/mg) | 36.4 ± 2.7 | 4.8 ± 0.1 | 3.6 ± 0.2 |
| | $K_m$ (mM) | 1.5 ± 0.2 | 6.9 ± 0.7 | 3.3 ± 0.6 |
| | $V_{max}/K_m$ | 24.3 | 0.7 | 1.1 |
| | $K_i$ (mM) | 21.0 ± 3.4 | – | – |
| Gl-7-ADCA | $V_{max}$ (U/mg) | 32.6 ± 1.2 | 5.1 ± 0.1 | 11.6 ± 0.4 |
| | $K_m$ (mM) | 1.3 ± 0.1 | 14.1 ± 1.3 | 1.5 ± 0.2 |
| | $V_{max}/K_m$ | 25.1 | 0.4 | 7.7 |
| | $K_i$ (mM) | 58.7 ± 6.0 | – | – |
| Succinyl-7-ACA | $V_{max}$ (U/mg) | 21.1 ± 1.9 | 4.9 ± 0.1 | 1.9 ± 0.04 |
| | $K_m$ (mM) | 2.8 ± 0.5 | 5.4 ± 0.5 | 2.8 ± 0.3 |
| | $V_{max}/K_m$ | 7.5 | 0.9 | 0.7 |
| | $K_i$ (mM) | 23.9 ± 4.7 | – | – |
| Adipyl-7-ACA | $V_{max}$ (U/mg) | 11 ± 1.5 | 2.2 ± 0.05 | 0.30 ± 0.01 |
| | $K_m$ (mM) | 7.5 ± 0.5 | 4.3 ± 0.3 | 4.4 ± 0.2 |
| | $V_{max}/K_m$ | 1.5 | 0.5 | 0.08 |
| | $K_i$ (mM) | 112 ± 15 | – | – |
| Adipyl-7-ADCA | $V_{max}$ (U/mg) | 6.3 ± 0.2 | 6.3 ± 0.1 | 0.80 ± 0.02 |
| | $K_m$ (mM) | 0.70 ± 0.06 | 7.9 ± 0.5 | 3.8 ± 0.4 |
| | $V_{max}/K_m$ | 9.0 | 0.8 | 0.2 |
| | $K_i$ (mM) | 127 ± 15 | – | – |
| Pentanoyl-7-ACA | $V_{max}$ (U/mg) | 2.2 ± 0.1 | 0.20 ± 0.05 | – |
| | $K_m$ (mM) | 17.4 ± 3.1 | 16.1 ± 0.8 | – |
| | $V_{max}/K_m$ | 0.1 | 0.01 | – |
| | $K_i$ (mM) | – | – | – |
| Cephalosporin C | $V_{max}$ (U/mg) | 0.7 ± 0.1 | 3.0 ± 0. 1 | – |
| | $K_m$ (mM) | 9.5 ± 0.3 | 12.2 ± 0.9 | – |
| | $V_{max}/K_m$ | 0.08 | 0.24 | – |
| | $K_i$ (mM) | – | – | – |
| *C3 derivatives of glutaryl-7-ACA/ADCA* | | | | |
| Gl-3Cl-7-ADCA | $V_{max}$ (U/mg) | 28.8 ± 0.8 | 5.9 ± 0.2 | 10.8 ± 0.4 |
| | $K_m$ (mM) | 1.0 ± 0.1 | 8.7 ± 0.8 | 2.2 ± 0.3 |
| | $V_{max}/K_m$ | 28.5 | 0.7 | 4.9 |
| | $K_i$ (mM) | 203 ± 27 | – | – |
| Gl-7-ZACA | $V_{max}$ (U/mg) | 2.6 ± 0.1 | 1.5 ± 0.1 | 28.3 ± 5.1 |
| | $K_m$ (mM) | 2.5 ± 0.3 | 3.0 ± 0.1 | 5.4 ± 1.5 |
| | $V_{max}/K_m$ | 1.0 | 0.5 | 5.2 |
| | $K_i$ (mM) | – | – | 22.8 ± 8.4 |

substrate (≈9-fold comparing wild-type and H296S-H309S VAC, Table 2). Indeed, wild-type VAC was inhibited at a Gl-3Cl-7-ADCA concentration ≥20 mM (Fig. 3B), a phenomenon that significantly distinguished it from the other two enzymes under investigation.

The introduction of a bulky side chain at position 3 – as using Gl-7-ZACA as a substrate – significantly affected the maximal activity of VAC enzymes (up to 14-fold lower for the wild-type VAC as compared to Gl-7-ACA, Table 2): the kinetic efficiency of GAR was about 5-fold and 10-fold higher than that of wild-type and H296S-H309S VAC, respectively, mainly because of a higher $V_{max}$ value. However, the higher activity of GAR on Gl-7-ZACA as compared to the standard substrate Gl-7-ACA was coupled to a substrate inhibition effect observed at substrate concentrations >10 mM (Fig. 3C).

## 4. Discussion

Semisynthetic cephalosporins are the most widely used antibiotics and are primarily produced from the intermediate 7-ACA, which is currently obtained by chemical deacylation or enzymatic conversion of the natural antibiotic CephC [8,12–15]. According to their substrate specificity and sequence conservation, known glutaryl acylases have been divided into different classes (see Section 1 and [9,10]). Here, we report a comparison of substrate preference among a commercially available GA (largely employed industrially to convert Gl-7-ACA into 7-ACA), the only known true CA (i.e. the H296S-H309S VAC variant, a glutaryl acylase which preferentially uses CephC), and the wild-type VAC enzyme.

A preliminary screening analysis performed using a simple colorimetric assay showed that both wild-type and H296S-H309S VAC enzymes did not efficiently use amino acid derivatives of glutaric acid, as well the corresponding methyl esters and amides. On the contrary, GAR was reported to efficiently hydrolyze these compounds [18]. These results were basically confirmed using the pH-Stat assay. All the three enzymes also showed a stereoselectivity preference: the pH-Stat measurements demonstrated a lower activity on glutaryl-D-phenylglycine methylester as compared to the corresponding L-isomer. Indeed, VAC and GAR enzymes resembled the P130 acylase (a member of class I glutaryl acylase according to [10]) that was recently reported to hydrolyze glutaryl-glycine or glutaryl-tryptophan with a kinetic efficiency ≈30-fold lower as compared to the reference substrate Gl-7-ACA [27].

Despite the practical significance of the information achieved by these studies – i.e. the possibility of selecting the best performing biocatalyst for each specific substrate – the fixed assay conditions did not allow to evaluate subtle differences among the studied enzymes. Accordingly, the kinetic parameters were determined

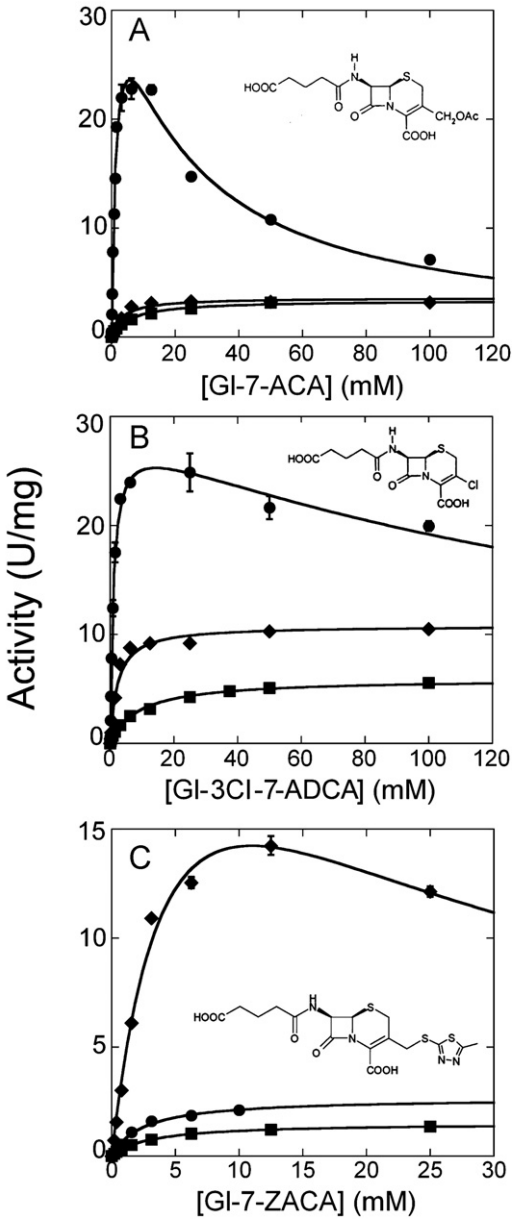


**Fig. 3.** Dependence of the activity values for the reaction of wild-type VAC (●), H296S-H306S VAC (■), and GAR (♦) from the concentration of different cephalosporin derivatives differing at C3. The data points were obtained as described in Fig. 2 using different enzyme concentrations (wild-type VAC: 3.8 or 89 μg/assay; H296S-H306S VAC: 57 or 108 μg/assay; GAR: 50 or 200 μg/assay). Panel (A) Gl-7-ACA; (B) Gl-3-Cl-7-ADCA; (C) Gl-7-ZACA. The curves are from fits based on the classical Michaelis–Menten equation or its modification to account for a substrate inhibition effect [25,26].

on the best cephalosporin derivatives identified in the preliminary analysis (Table 2). The acylases under investigation did not show a preference between the ACA vs. ADCA nucleus, while the length of the substituent in C7 represented a main constrain for substrate recognition. For all the enzymes the highest maximal activity was observed with compounds having a 4-carbons side chain linked to the C═O—NH-group in C7. In fact: (i) a shorter side chain (i.e. succinyl-7-ACA) significantly affected the kinetic properties for both GAR and wild-type VAC, this resembling what has been reported for P130 acylase (its kinetic efficiency was 36-fold lower with succinyl-7-ACA vs. Gl-7-ACA) [27]); (ii) for all the acylases under investigation a longer side chain in C7 drastically affected the activity as compared to Gl-7-ACA – and similarly no activity was detected for the P130 acylase with adipyl-7-ACA [27]; (iii) the activity was fully lost for GAR with the neutral pentanoyl-7-ACA and drastically (≈20-fold) decreased for the VAC enzymes; (iv) the H296S-H309S VAC variant only showed a significant activity and kinetic efficiency on CephC, which C7-side chain contains a D-amino adipic acid. The docking analysis of CephC to the active site of VAC was of help to rationalize the effect of the substituent in C7 on its kinetic properties: this analysis showed that the substituent in C3 pointed toward the active site entrance while the side chain in C7 was located into the inner part, thus being more susceptible to hindering effects [21]. In order to improve the activity of SY-77 GA on adipyl-7-ADCA, region- and site-saturation mutagenesis were previously used [28,29]. However, the best enzyme variant identified, containing 3-point substitutions, showed a substantial lower efficiency than wild-type VAC ($k_{cat}$ = 1.2 $s^{-1}$ vs. 8.7 $s^{-1}$ and $K_m$ = 0.8 vs. 0.7 mM, respectively).

Interestingly, when a bulky substituent was introduced at position C3 of the "cephem" a different effect on the kinetic properties of the employed enzymes was observed. A significant decrease in maximal activity was apparent on Gl-7-ZACA as compared to the reference substrate Gl-7-ACA for the VAC enzymes, while a ≈10-fold increase was evidenced for GAR.

In summary, we have clarified the substrate preference of two prototypical enzymes for GA vs. CA subfamilies, of main industrial relevance. These achievements pave the way to the ability of choosing the best enzyme to be used for the hydrolysis of different compounds of industrial importance.

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