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Kinetic resolutions of racemic amines and alcohols catalyzed by an industrial glutaryl-7-aminocephalosporanic acid acylase with unexpected broad substrate specificity

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Abstract—An industrial glutaryl-7-aminocephalosporanic acid acylase (GAR) possesses a significant broad substrate specificity that crosses over the usual cephalosporanic skeleton. Enantioselective amidase and even esterase activities have been observed with all the glutarates of racemic substrates investigated, with a stereopreference for the (*S*)-enantiomer. The different physical–chemical properties of reagents and products allow their easy separation by solvent extraction, avoiding cumbersome chromatography or distillation processes during reaction work-up.

1. Introduction

Enzymatic kinetic resolution (EKR) of racemates is, by far, the most investigated application of biocatalysis as testified by the number of papers that have been published on this topic, mainly describing the performances of hydrolases in water or in organic solvents.[1–3]

A practical drawback that is often encountered applying EKR is related to reaction work-up: usually reagents and products have very similar chemical–physical properties and, as a consequence, their separation requires careful chromatographic or fractional distillation steps, thus severely hampering the scale-up of most of these transformations. To overcome this problem in the EKR of racemic carboxylates, we have proposed the use of amino-alcohols as nucleophiles in transesterification reactions performed in organic solvents:[4,5] the product could be easily separated from the unreacted enantiomer by selective extraction with mildly acidic water solutions. In the present work this approach has been extended to the EKR of racemic amines and alcohols.

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2. Results and discussion

2.1. Glutaryl acylases

Glutaryl-7-aminocephalosporanic acid acylases (GAs) are enzymes that catalyze the cleavage of the amide bond of the glutaryl-7-aminocephalosporanic acid (Glu-7-ACA) **1** side chain to give 7-aminocephalosporanic acid (7-ACA) **2**. These enzymes are industrially used in a two-step ton-scale biocatalyzed process that produces **2** from cephalosporin C **3**.[6,7]

Very few reports are available in the literature on the activity of GAs towards substrates other than **2**.[8,9] These data are limited to a small number of cephalosporin derivatives carrying either a side chain linked at N-7 (R, Scheme 1) different from glutarate or a methyl group instead of an acetate in C-3 (R′ = Me, derivatives of 7-ADCA, **4**). More recently, an investigation on the synthetic activity of GA in organic solvents and in heterogeneous substrate mixtures has been presented, with some additional indications on GA substrate specificity.[10]

A recent paper has described the structure of the active site of a GA from *Pseudomonas diminuta* KAC-1 in complex with **1**.[11] X-Ray analysis of the crystallized protein identified three substrate moieties that are spe-

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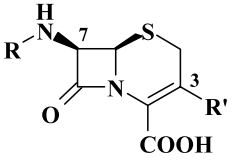
cifically recognized by this specific enzyme: the glutaric chain, the β-lactam nucleus and the substituent in C-3 (acetate in **1**).

2.2. Investigation of GA substrate specificity

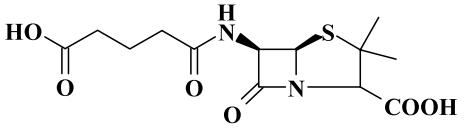
Although these data seemed to indicate a very strict substrate specificity of these enzymes, we decided to investigate the substrate specificity of an industrial GA commercialized by Recordati SpA (GAR), an enzyme whose performances towards **1** have been previously studied in detail.[12] Several amides of 7-ACA and 7-ADCA were prepared by reaction of **2** or **4** with a suitable anhydride. The glutarate moiety was by far the preferred one and, additionally, no conversion was observed with substrates carrying a neutral aliphatic side chain (Table 1).

On the other hand, the C-3 position was much less crucial for the GAR enzyme, and glutaryl derivatives of 7-ACA carrying different C-3 substituents were all efficiently hydrolyzed (data not shown). We also found that the presence of the cephalosporanic skeleton was not a strict requirement, as glutaryl 6-aminopenicillanic acid **14** was also an excellent substrate (118% relative rate of hydrolysis compared to **1**) for the enzyme.

As the third interaction site of **1** with the GA from *Pseudomonas diminuta* was reported to be the β-lactam ring,[11] a logical further step for our investigation was the modification of this part of the molecule. Accord-



1 : R = HOOC-$(CH_2)_3$-CO ; R' = CH_2OAc
2 : R = H ; R' = CH_2OAc
3 : R = HOOC-$CHNH_2$-$(CH_2)_3$-CO ; R' = CH_2OAc
4 : R = H ; R' = CH_3 (7-ADCA)
5 : R = HOOC-$(CH_2)_3$-CO ; R' = CH_3
6 : R = HOOC-$(CH_2)_2$-CO ; R' = CH_2OAc
7 : R = HOOC-$(CH_2)_4$-CO ; R' = CH_2OAc
8 : R = CH_3-$(CH_2)_3$-CO ; R' = CH_2OAc
9 : R = CH_3-$(CH_2)_2$-CO ; R' = CH_2OAc
10 : R = HOOC-$(CH_2)_2$-CO ; R' = CH_3
11 : R = HOOC-$(CH_2)_4$-CO ; R' = CH_3
10 : R = CH_3-$(CH_2)_3$-CO ; R' = CH_3
13 : R = CH_3-$(CH_2)_2$-CO ; R' = CH_3



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Scheme 1. Structures of cephalosporanic and penicillinic amides.

Table 1. Relative rates of hydrolysis of cephalosporanic amides[a]

Compound	Relative rate[b]
1	100.0
6	2.2 ± 0.4
7	6.9 ± 0.3
8	0
9	0
5	111.1 ± 1.6
10	26.9 ± 0.9
11	8.3 ± 0.1
12	0
13	0
14	118.0 ± 0.1

[a] Typical procedure for the synthesis of cephalosporanic amides. 7-ACA or 7-ADCA (5 mmol) were dissolved in 20 mL of 1 M $NaHCO_3$. The anhydrides (1 equiv.) were dissolved in 5 mL acetone and the two solutions were mixed and let to react for 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. Products structures were confirmed by ^1H NMR.

[b] Reactions conditions: Total volume, 20 mL: 50 mM substrate in H_2O, 0.22 U/mL GAR (1 Unit is defined as the amount of GAR that hydrolyzes 1 μmol of **1** per minute at pH 8 and at 25°C. The specific activity of the GAR sample used in this work was 2.28 U/mg). Reaction solutions were stirred at 25°C in a 718 STAT Titrino automatic titrator (Metrohm Ltd) maintaining a constant pH value (8.0) by adding 0.1 M NaOH. Experiments were repeated in duplicate at least. The rates of hydrolysis were calculated from the amount of NaOH solution added in the time unit. The rate of hydrolysis of compound **1** (4.4 μmol/min) was taken as 100.

Table 2. Relative rates of hydrolysis of amino acids glutaryl amides

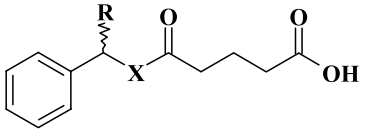
Compound	Relative rate[a]
N-Glutaryl glycine methyl ester	9.7 ± 0.2
N-Glutaryl-L-alanine methyl ester	25.0 ± 0.6
N-Glutaryl-D-alanine methyl ester	2.2 ± 0.2
N-Glutaryl-L-alanine	2.7 ± 0.2
N-Glutaryl-D-alanine	< 0.1[b]
N-Glutaryl-L-phenylalanine methyl ester	15.7 ± 2.0
N-Glutaryl-D-phenylalanine methyl ester	4.5 ± 0.2
N-Glutaryl-L-phenylalanine	2.5 ± 0.1
N-Glutaryl-D-phenylalanine	2.0 ± 0.4
N-Glutaryl-L-phenylglycine methyl ester	59.3 ± 1.0
N-Glutaryl-D-phenylglycine methyl ester	0.23 ± 0.01[b]
N-Glutaryl-L-phenylglycine	7.5 ± 0.8
N-Glutaryl-D-phenylglycine	1.1 ± 0.2

[a] Reactions conditions: Total volume, 20 mL: 50 mM substrate in H_2O, 0.22 U/mL GAR. Reaction solutions were stirred at 25°C in a 718 STAT Titrino automatic titrator (Metrohm Ltd) maintaining a constant pH value (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. Experiments were repeated in duplicate at least. The rate of hydrolysis of compound **1** (4.4 μmol/min) was taken as 100.0.

[b] Same reaction conditions but 0.44 U/mL GAR and 0.025 M NaOH.

ingly, we synthesized a series of glutaryl amides of amino acids and of their methyl esters. The relative rate of hydrolysis catalyzed by GAR, reported in Table 2, allowed us to draw the following important conclusions: (a) the presence of a β-lactam moiety is not essential for enzyme activity: all the glutaryl amino acids investigated were substrates for GAR. (b) monomethyl esters of *N*-glutaryl amino acids were better substrates than the corresponding free acid derivatives. (c) GAR displayed a significant enantio-preference for the glutaryl amides of L-amino acids, a property that is not in contrast with the 'usually' catalyzed hydrolysis of the glutaryl moiety linked at the cephalosporin 7-position (originally the α-amino group of a L-cysteine).

Stimulated by these positive results, substrate modification was pushed further and glutarates of racemic amines (i.e. **15**–**16**, Scheme 2) and even of racemic alcohols (i.e. **17**–**18**) were considered. We were pleased to find that both these sets of compounds were hydrolyzed by GAR (thus showing, for the first time, that this enzyme also possesses a significant esterase activity) with an appreciable preference for the (*S*)-enantiomers. Table 3 reports the *E* values[13] obtained with a homogeneous set of compounds. Worth noting is the chemical-physical difference between the substrates (water-soluble carboxylates) and the products (alcohols or amines), that therefore could be separated either by direct solvent extraction (alcohols) or by selective extraction following pH-adjustment (amines).



15 : R = $COOCH_3$; X = NH
16 : R = CH_3 ; X = NH
17 : R = $COOCH_3$; X = O
18 : R = CH_3 ; X = O

Scheme 2. Structures of compounds **15**–**18**.

Table 3. Enantioselectivity of GAR towards glutaryl amides and esters

Compound	Conv. (%)[a]	e.e.$_{Prod}$	*E*
15	47[b]	95[d]	104.4
16	43[b]	78[d]	14.6
17	50[c]	62[c]	7.9
18	48[d]	58[d]	6.3

[a] Reactions conditions: 50 mM substrate in H_2O, 2 U/mL GAR. Reaction solutions were stirred at 25°C in a 718 STAT Titrino automatic titrator (Metrohm Ltd) maintaining a constant pH value (7.0) by adding 0.1 M NaOH. Conversion and e.e.$_{Prod}$ were evaluated by chiral column HPLC.

[b] Chirobiotic T column; **15**: eluent MeOH–TEAA, 20:80 (TEAA = H_2O–Et_3N–AcOH 100:0.1:0.1, pH 4.1), flow rate 0.9 mL/min; **16**, eluent MeOH–TEAA, 10:90, flow rate 1 mL/min.

[c] Chiralcel OJ column; **17**, eluent hexane–*i*PrOH–HCOOH, 9:1:0.1, flow rate 0.8 mL/min.

[d] Chiralcel OD column; **15**, eluent hexane–*i*PrOH–Et_2NH, 95:5:0.1, flow rate 0.5 mL/min; **16**, eluent hexane–*i*PrOH–Et_2NH, 95:5:0.1, flow rate 1 mL/min; **18**, eluent hexane–*i*PrOH–CF_3COOH, 98:2:0.1, flow rate 0.75 mL/min.

Reactions with compounds **15**–**18** were scaled up. In a typical experiment 1.5 g (5 mmol) of **15** were dissolved in 100 mL H_2O. The pH was adjusted to 7.0 and the reaction was started by adding 200 U of GAR and monitored by chiral column HPLC (see Table 3), keeping the pH constant by adding 0.1 M NaOH via an automatic titrator. The reaction was stopped at 47% conversion (approximately 2 h) by adjusting the pH to 3.0 with AcOH. The solution was extracted with 50 mL AcOEt (three times) to remove the unreacted **15** and glutaric acid. The water phase was adjusted to pH 7, freeze dried and lyophilized to give pure L-phenylglycine methyl ester with 95% e.e.

GAR was also used immobilized on a solid support:[12] the enzyme was recovered by filtration and reused for 4 times without showing appreciable loss of activity.

3. Conclusions

To summarize, we have demonstrated that an industrial GA possesses a significant broad substrate specificity that crosses over the cephalosporanic skeleton. Enantioselective amidase and even esterase activities have been observed with all the racemic glutarate substrates investigated. The different physical–chemical properties of reagents and products allow their easy separation by solvent extraction, avoiding chromatography or distillation during work-up. Additionally, syntheses of glutaryl amides and esters are trivial and require a very cheap and available acylating agent.

Finally, it is worth noting that the GA used in this study is an industrial enzyme expressly developed for the efficient hydrolysis of Glu-7-ACA **2**. The increasing numbers of GAs that are becoming available,[8,9] as well as the modification of the existing enzymes by protein engineering (site-directed[14] and/or random mutagenesis) might offer soon new GAs with improved activity and enantioselectivity towards target racemic substrates.

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