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Glutaryl-7-ACA acylase catalyses the synthesis of amide bond in heterogeneous substrate mixtures

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Abstract

Covalently immobilised glutaryl acylase (GA), used in industry for the hydrolysis of glutaryl-7-aminocephalosporanic acid, was employed for the first time in low-water media. Results indicate that the enzyme catalyses the synthesis of the amide bond when working at very high substrate concentrations, namely in heterogeneous substrate mixtures, whereas no enzymatic activity was detected using the enzyme in diluted organic solvent solutions. Preliminary data on GA substrate selectivity suggest that a negative charge on the acyl moiety is crucial for substrate recognition.

Keywords: Glutaryl-7-ACA acylase; Heterogeneous substrate mixture; Organic solvent; Synthesis of amide bond; Low-water media

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1. Introduction

Glutaryl-7-ACA acylase (GA) is an industrially relevant enzyme since it is employed in the two step-enzymatic deacylation of cephalosporin C (CPC) to 7-aminocephalosporanic acid (7-ACA) which is the primary building block for the synthesis of semi-synthetic cephalosporin antibiotics. The enzyme is responsible for the hydrolysis of glutaryl-7-ACA, which results from the conversion of CPC to 2-ketoadipoyl-7-ACA catalysed by a D-amino acid oxidase with the consequent formation of H_2O_2 that causes spontaneous decarboxylation [1].

GA belongs to the superfamily of the *N*-terminal nucleophile hydrolases, which includes also penicillin G acylase (PGA) [2]. The catalytic serine of GA (Ser B1) is located in a *N*-terminal position with important consequences on its mechanism of action [3] as well as on its proteolytic activation [4].

A recent study has reported the characterisation of one commercial preparation of GA [1] available as covalently immobilised form on polyacrylamidic support, which is employed for the industrial production of 7-ACA. However, the few studies on GA present in the literature concern mainly the activity of this biocatalyst towards glutaryl-7-ACA and cephalosporin C in the perspective of improving its industrial exploitation in hydrolytic processes. Up to date very few or no data are available neither on substrate selectivity of GA nor on its activity in catalysing the synthesis of amide bonds. Recent structural studies suggest that, likewise PGA, this enzyme is endowed with high substrate selectivity as far as the acyl moiety concerns [3]. Such novel selective enzyme with amidase/acylase activity could be potentially exploited, as in the case of PGA, in selective protection/deprotection of functional groups as well as in asymmetric synthesis [5–7]. Therefore, here we present the first study on

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GA synthetic potential in low-water media to provide preliminary guidelines for the employment of this biocatalyst in organic synthesis.

2. Experimental

2.1. Materials

Glutaryl-7-ACA acylase was kindly donated by Recordati (Milano-Italy). The enzyme is covalently immobilised on polyacrylamide beads [1] and stored in glycerol. GA was rinsed with water and filtered in order to remove the glycerol and finally it was partially dehydrated. Dehydration by lyophilisation and at reduced pressure led to water contents, respectively of 3 and 8%. Dehydration by means of Celite R-640® in petroleum ether was performed according to [8], obtaining a water content of 22%. Residual water was measured by Karl Fischer titration (Mettler).

Celite R-640® is from FLUKA and was dried at 180 °C for 24 h prior to use. *p*-Nitroaniline and glutaric anhydride are from ALDRICH, L-glutamyl-5-(*p*-nitroanilide) is from BioChemika, L-tyrosine ethyl ester, L-tyrosinamide, L-phenylglycine methyl ester as the hydrochloride, glutaric acid dimethyl ester, glutaric acid and glutamic acid, are from SIGMA. L-Phenylglycine methyl ester free base was prepared according to [6].

Glutaric acid methyl ester **2** was synthesised according to [9] and characterised by IR, ^{1}H NMR and ^{13}C NMR (Varian-Gemini 200 spectrometer at 200 MHz). Amides **5**, **6**, **9** and **10** were synthesised according to [10]. Amides **7** and **8** were synthesised according to [11]. All amides were characterised by ^{1}H NMR, ^{13}C NMR and ES-MS (API1 Perkin-Elmer SCIEX mass spectrometer).

2.2. Assay of GA hydrolytic activity

Hydrolytic activity of covalently immobilised GA after different dehydration treatments was evaluated by measuring initial rates of enzymatic hydrolysis of 3 ml of 1×10^{-3} M solution of glutaryl-*p*-nitroanilide in 0.1 M phosphate buffer pH 8 in the presence of 100 mg of GA. Release of *p*-nitroaniline was calculated on the basis of the increase in adsorbance at 410 nm (UV–VIS spectrophotometer Perkin-Elmer Lambda 5). Calibration curves were built up on the basis of nine solutions of *p*-nitroaniline at different concentrations between 1 and 30×10^{-5} M.

2.3. RP–HPLC analysis

A column Spherisorb C18 ODS1 Waters (4.6 mm × 250 mm) was employed and products were eluted using mixtures of water/acetonitrile with the addition of 0.025% TFA. Detector: UV–VIS spectrophotometer.

2.4. Reactions in organic solvents

An amount of 100–200 mg of pre-treated GA were added to 1 ml of 160 mM solutions of the reactants and incubated at 30 °C in an orbital mixer (Darai, Trieste-Italy) at 180 rpm. Withdrawals were analysed by HPLC and ES-MS. The formation of the salt between ester **2** and amine **4** was confirmed by ^{1}H NMR.

2.5. Reactions in heterogeneous substrate mixture

One hundred sixty micromoles of the solid amines were added to the enzyme as specified in Table 1. Mixtures were placed in a 5 ml vial and the reactions were started by the addition of 640 μmol of the liquid diester **1**. The suspension was mixed and then thermostatted at 30 °C during the reaction course. Withdrawals were suspended in acetonitrile, sonicated and centrifuged. The organic liquid phase was diluted with water/TFA 0.025% and analysed by RP-HPLC. Elution conditions of amides **5**–**8**: acetonitrile/water (70:30). Products were identified by comparison with chemically synthesised standards. Moreover, peaks were collected and analysed by ES-MS. Amide **5** was isolated from a larger scale experiment set up using 1.12 mmol of amine **3**, 2.1 g of GA and 4.50 mmol of ester **1**. After 38 h the reaction was stopped by adding 20 ml of dry methanol and removing the enzyme by filtration on filter paper. The GA was rinsed with further 10 ml of methanol and the filtrated was concentrated at reduced pressure. The resulting oil was washed with 10 ml of petroleum ether, dissolved in 15 ml of dichloromethane and extracted with 5 ml of a 0.1N solution of HCl. The amide **5** was finally extracted from the aqueous phase with ethyl acetate (5 ml twice) that was evaporated at reduced pressure. The oily product **5** was characterised by ^{1}H NMR.

Table 1
Synthesis of amides **5** and **6** catalysed by covalently immobilised GA using amines **3** and **4** (160 μmol) suspended in ester **1** (640 μmol)

GA[a] (mg)	Temperature (°C)	Amine	Water[b] (μl)	Final conversion (%)	Amide
80	30	**3**	18	11	**5**
300	30	**3**	66	48[c]	**5**
80	30	**4**	18	16	**6**
80	40	**4**	18	7	**6**
300	40	**4**	66	24	**6**

[a] GA covalently immobilised and partially dehydrated to 22% water content by using Celite R-640® in petroleum ether (see Section 2).

[b] Residual water of the enzymatic preparations after partial dehydration measured by means of Karl Fischer titration.

[c] Yield of **5** reached in 96 h, after two additions of 25 μl each of water at time 28 and 72 h. Conversions after 24 and 48 h were 31 and 44%, respectively.

In order to verify the enzymatic hydrolysis of diester **1** to ester **2** the following experiment was performed: 200 mg of GA and 198 μmol of **1** were mixed together and incubated at 30 °C for 24 h. To point out the formation of the corresponding carboxylic acid, 1.5 ml of D_2O were added to the reaction mixture and the solution was analysed by ^1H NMR after removing the GA by filtration. A blank experiment was carried out by incubating the diester **1** in the presence of an amount of water (44 μl) calculated on the basis of the water content of GA employed in the previous experiment.

2.6. Enzymatic synthesis of amide **5** in aqueous media

Ninety milligrams of GA and 24 mg (160 mM) of monoester **2** or 26 μl (160 mM) of diester **1** were added to a 320 mM solution of amine **3** in 0.1 M phosphate buffer pH8. The reaction was thermostatted at 30 °C for 24 h and then analysed by RP-HPLC. The peak corresponding to amide **5** was compared to a chemically synthesised standard and finally collected and analysed by ES-MS.

2.7. Determination of initial rates of enzymatic hydrolysis of amides **6**, **7** and **9**

Enzymatic hydrolysis was carried out by adding 100 mg of enzyme to 3 ml of 1×10^{-2} M solutions of amides in 0.1 M phosphate buffer pH 8, at 30 °C. Initial rates were determined by following the first 10% of conversion determined quantitatively by RP-HPLC. Mobile phase: acetonitrile/water (50:50). UV detector: $\lambda_{max} = 275$ nm (amides **6**, **7**); $\lambda_{max} = 260$ nm (amide **9**). Calibration curves were built up on the basis of seven solutions of the corresponding amines at concentrations between 1 and 10×10^{-4} M.

2.8. Determination of initial rates of enzymatic hydrolysis of amides **10** and **11**

One hundred milligrams of enzyme were added to 3 ml of 1×10^{-3} M solutions of amides in 0.1 M phosphate buffer pH 8 and incubated at 30 °C. Release of the corresponding amines was followed by UV spectroscopy at 410 nm as specified in Section 2.2.

2.9. Measurement of water activity

a_w was measured according to [12] using a hygrometer (Darai, Trieste-Italy).

2.10. ^1H NMR characterisation

Glutaryl-L-tyrosinamide **5** (chemical synthesis): ^1H NMR (200 MHz, DMSO): 1.61 (m, 2H, $CH_2\mathbf{CH_2}CH_2$); 2.03 (m, 2H, $\mathbf{CH_2}CO$); 2.15 (t, 2H, $HOOC\mathbf{CH_2}$); 2.70 (m, 2H, $\mathbf{CH_2}Ph$); 4.27 (m, 1H, $NHC\mathbf{H}$); 6.49 (d, 2H, **Ph**); 6.97 (d, 2H, **Ph**); 7.36 (s, 2H, $CON\mathbf{H_2}$); 7.89 (d, 1H, $CON\mathbf{H}$).

Glutaryl-L-tyrosinamide **5** (enzymatic synthesis): ^1H NMR (200 MHz, DMSO): 1.64 (m, 2H, $CH_2\mathbf{CH_2}CH_2$); 2.06 (m, 2H, $\mathbf{CH_2}CO$); 2.20 (t, 2H, $HOOC\mathbf{CH_2}$); 2.70 (m, 2H, $\mathbf{CH_2}Ph$); 4.29 (m, 1H, $NHC\mathbf{H}$); 6.59 (d, 2H, **Ph**); 6.97 (d, 2H, **Ph**); 7.37 (s, 2H, $CON\mathbf{H_2}$); 7.90 (d, 1H, $CON\mathbf{H}$).

Salt formed in hydrophobic organic solvent between amine **4** and monoester **2**: ^1H NMR (200 MHz, DMSO): 1.40 (t, 3H, $COCH_2\mathbf{CH_3}$); 1.63 (m, 2H,

$CH_2C\mathbf{H_2}CH_2$); 2.20 (m, 2H, $C\mathbf{H_2}COOH$); 2.25 (t, 2H, $C\mathbf{H_2}COOCH_3$); 2.65 (dd, 2H, $C\mathbf{H_2}Ph$); 3.42 (t, 1H, $NHC\mathbf{H}$); 3.55 (s, 3H, $C\mathbf{H_3}OCO$); 3.98 (q, 2H, $COC\mathbf{H_2}CH_3$); 6.61 (d, 2H, **Ph**); 6.90 (d, 2H, **Ph**).

Enzymatic hydrolysis of diester **1**: reaction mixture: ^1H NMR (200 MHz, D_2O): 1.69 (m, 2H, $CH_2C\mathbf{H_2}CH_2$); 2.08 (t, 0.3H, $C\mathbf{H_2}COOH$); 2.24 (t, 3.7H $C\mathbf{H_2}COOCH_3$); 3.48 (s, 5.6H, $C\mathbf{H_3}OCO$).

Hydrolysis of diester **1**: Blank experiment in the absence of enzyme. Reaction mixture: ^1H NMR (200 MHz, D_2O): 1.71 (m, 2H, $CH_2C\mathbf{H_2}CH_2$); 2.26 (t, 4H, $C\mathbf{H_2}COOCH_3$); 3.50 (s, 6H, $C\mathbf{H_3}OCO$).

3. Results and discussion

A covalently immobilised GA, employed industrially, was used in organic solvents and in heterogeneous substrate mixtures to investigate its activity in catalysing the synthesis of amide bonds. The enzyme is provided by the producer as a suspension in glycerol, so that it was firstly rinsed with water and finally partially dehydrated to make it suitable for application in low-water media.

Three different dehydration methods were compared and, as previously observed also for PGA [8], removal of water by means of Celite R-640® in petroleum ether (22% final water content) led to the most active enzymatic preparation. As a matter of fact, dehydration either by lyophilisation or under reduced pressure caused a dramatic irreversible loss of enzymatic activity (decrement of 75%), evaluated as hydrolytic activity in aqueous buffer towards glutaryl-*p*-nitroanilide (see Section 2).

The enzyme was tested in solvents differing for physical–chemical properties such as *tert*-amyl alcohol, dichloromethane, *n*-hexane, acetonitrile, toluene. The pre-treated biocatalyst, suspended in organic solvents, gave at the equilibrium values of water activity ranging from 0.73 and 0.89. Acetonitrile/water mixtures at $a_w > 0.95$ were also employed. Esters **1** and **2** (160 mM) were used as acylating agents in the presence of amines **3** and **4** (160 mM). It was verified (^1H NMR) that monoester **2**, due to the free carboxy group, causes the protonation of the amines and the precipitation of the corresponding salts. However, no product was detected even when diester **1** was employed.

It has been demonstrated that large space-time and product yields can be obtained in biotransformations of aqueous or organic suspensions with solid substrates and/or products [13]. On the light of the lack of activity of GA in diluted organic solvent solutions, the reaction system was modified by using reaction mixtures consisting mainly of solid reactants suspended in a low volume, saturated liquid phase [13]. Diester **1** is liquid at 30 °C, so that it was employed as a micro-liquid phase that partially dissolved the solid amines L-tyrosinamide (**3**) and L-tyrosine ethyl ester (**4**). Moreover, the reaction mixtures contained variable amounts of water, as specified in Table 1, due to the residual hydration of the biocatalyst.

Despite the fact that amines remained mainly in the form of a suspension and reaction mixtures appeared to be very viscous and poorly solvated, enzymatic synthetic activity was detected. In this type of system the synthesis occurs in the microscopic liquid phase around the enzyme and the high substrate concentration improves the biocatalyst efficiency in comparison with conventional enzymatic reaction in organic solvents [14].

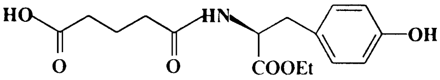
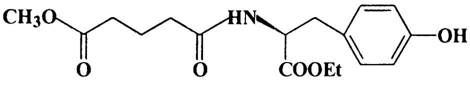
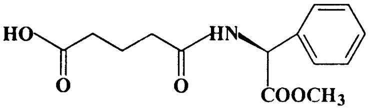
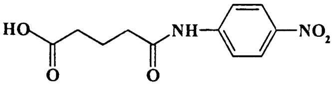
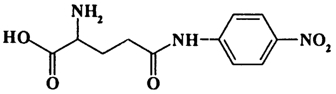
Table 1 reports reaction conditions and conversions achieved with experiments performed in heterogeneous substrate mixtures. Unexpectedly, reactions carried out with diester **1** led, after 24 h, to the formation, respectively of amides **5** and **6** which present the free carboxy group in the ω-position, whereas the formation of amides **7** and **8** (7 and 13%, respectively) was observed only at very long reaction times (2 weeks). This was confirmed by comparison with chemically synthesised standards as well as by ES-MS analysis of peaks eluted from HPLC.

Formation of amides **5** and **6** apparently stopped after 24 h and conversion did not exceed 31%, except when small volumes of water were added during the course of the reaction (25 μl each, after 28 and 72 h, see footnote c, Table 1). This allowed the synthesis of **5** to proceed up to 48% conversion in 96 h.

Increasing the temperature did not translate in any positive effect. No product was observed when the monomethyl ester **2** was used, because, as previously observed in reactions performed in organic solvent, the free carboxy group causes the protonation of the amine component.

It is noteworthy that the solid state of the amine reactants does not represent a major limit to the achieve-

Table 2
Hydrolysis of amides of glutaric and glutamic acids catalysed by GA in phosphate buffer, pH 8 at 30 °C

Amide	v_o (μmol min^{-1} mg enzyme^{-1})[a]
 **6**	0.20[b]
 **7**	n.d. [b,c]
 **9**	0.11[b]
 **10**	0.025[d]
 **11**	0.023[d]

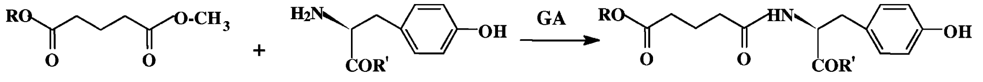
[a] Enzyme amount was calculated as dry weight.
[b] Three milliliter of 1×10^{-2} M solutions of amides in 0.1 M phosphate buffer pH 8, at 30 °C.
[c] Not detectable after 7 days.
[d] Three milliliter of 1 × 10^{-3} M solutions of amides in 0.1 M phosphate buffer pH 8, at 30 °C.

ment of high conversions. Indeed, L-phenylglycine methyl ester, which is liquid at 30 °C and is structurally similar to amines **3** and **4**, is also acylated by GA (result not shown) and with conversions (7% conversion after 24 h) comparable to those obtained by using solid amines.

In order to gain more insights into the mechanism of amine acylation catalysed by GA, hydrolytic reactions reported in Table 2 were performed.

Results indicate that, derivatives of glutaric and glutamic acids are readily hydrolysed by GA in phosphate buffer at pH 8, except for amide **7** that presents the esterified carboxy group which also remains intact even after 7 days of incubation.

This finding suggests that, in the enzymatic synthesis of amides **5** and **6**, the ω-ester bond must be hydrolysed enzymatically before the formation of the amide bond between glutaric acid and the amines. Indeed, when diester **1** was incubated in the presence of GA but with no amine, the ester bond hydrolysis was observed by ^{1}H NMR analysis of the reaction mixture, whereas no formation of carboxylic groups was



1 : R = CH_3
2 : R = H

3 : R' = NH_2
4 : R' = OC_2H_5

5 : R = H; R' = NH_2
6 : R = H; R' = OEt
7 : R = CH_3; R' = OC_2H_5
8 : R = CH_3; R' = NH_2

Scheme 1. Acylation of amines **3** and **4** catalysed by covalently immobilised GA in heterogeneous substrate mixture.

observed in the absence of the enzyme. These two results confirm that the hydrolysis of **1** is enzymatically catalysed.

Both hydrolytic and synthetic results obtained in this work can be interpreted on the light of a recent study reporting the three-dimensional structure of GA from *Pseudomonas diminuta* [3]. X-ray data have shown that GA has an active site with a side-chain binding pocket which is mostly hydrophobic but presenting, unlike PGA, also two hydrophilic residues (Gln and Arg). There are evidences that the selectivity of GA towards different side-chain acyl moieties is related to the presence of these two positively charged residues, which stabilise the negatively charged carboxy group of glutaric and glutamic acids through electrostatic interactions.

As a consequence, diester **1** is expected to be a poor substrate for GA because it lacks the negatively charged carboxy group. On the basis of these observations, we have formulated the hypothesis that in the acylation of amines **3** and **4** the residual water present on the biocatalysts allows the slow hydrolysis of diester **1** to monoester **2**. This allows the concentration of monoester **2** to be sufficiently low in the reaction system to prevent the protonation of amines, which eventually are acylated by GA using the monoester **2** as acyl-donor. Small additions of water during the course of the reaction force the hydrolysis of **1** to proceed, thus improving acylation yields Scheme 1.

It is noteworthy that the synthesis of amide **5** can be observed by performing the acylation of amine **3** in aqueous buffer at pH 8 (see Section 2.6) either using diester **1** or monoester **2** since in such experimental conditions the protonation of the amine is prevented.

Given the fundamental role of different charged functional groups involved in the mechanism of GA catalysed synthesis of amide bond, further studies are in progress to improve the enzyme performance by controlling the ionisation state of both the substrates and the biocatalyst.

4. Conclusions

This first study on GA application in low-water media points out the potential application of this industrial catalyst also in synthetic reactions. Covalently immobilised GA catalyses the acylation of amino groups in heterogeneous substrate mixtures, whereas no activity was detected in more diluted experimental conditions, namely in organic solvent. It is generally recognised that the catalytic activity displayed by enzymes in neat organic solvent is far lower than in water [15]. Working in heterogeneous substrate mixtures proved to be a powerful technique, particularly suitable for those biocatalysts, such as GA, which are poorly or no active on substrates in diluted organic solutions, and require high reactant concentration in order to be efficient in low-water media.

Preliminary data on substrate selectivity indicate that the negatively charged carboxy group of glutaric and glutamic acid is crucial for substrate recognition.

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