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Purification and Stability of Glutaryl-7-ACA Acylase from *Pseudomonas sp.*

EZIO BATTISTEL,* DANIELE BIANCHI, ROSSELLA BORTOLO, AND LUCIA BONOLDI

ENICHEM, Istituto Donegani, via Fauser 4—28100 Italy

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ABSTRACT

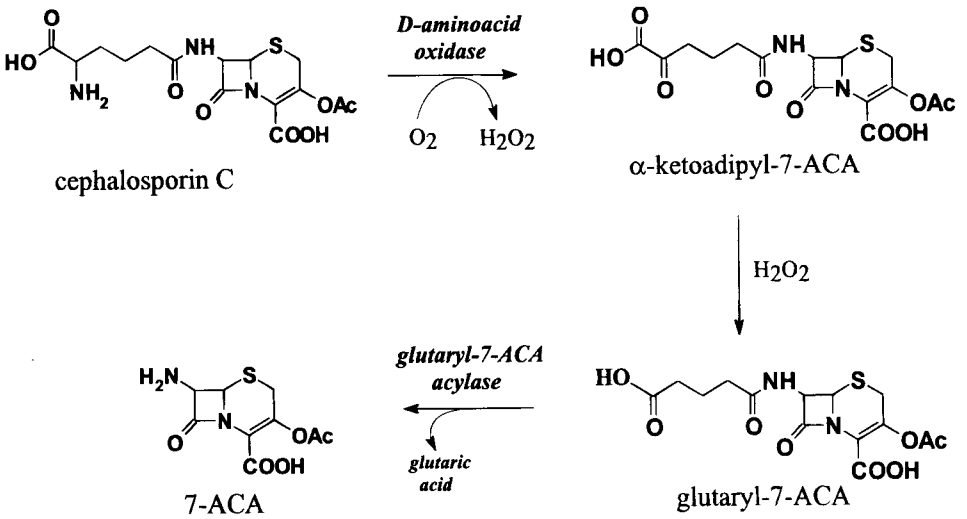
The enzyme glutaryl-7-ACA acylase from *Pseudomonas sp.* NCIMB 40474, produced by a recombinant *Escherichia coli* host, was purified to homogeneity. The enzyme is a tetramer composed of two couples of asymmetric dimers, each of them constituted of two subunits of mol wt 18 and 52 kDa, respectively. It was found that glutaric acid, one of the products of the substrate hydrolysis, is an effective acylase inhibitor. Between pH 6.0 and pH 10.0, the enzymatic activity is almost constant, but below pH 6.0 it progressively declines. The acylase activity decreased sharply as a function of guanidine HCl concentration. The loss is significant even at concentrations of denaturant lower than those causing unfolding, as suggested by UV spectroscopy and fluorescence emission studies. In these conditions (low denaturant concentration and low pH) the inactivation of the enzyme is caused by the tetramer dissociation into dimers. The lability of the quaternary structure of the enzyme is a key feature that must be taken into account for the improvement of the catalyst stability.

Index Entries: Glutaryl-7-ACA acylase; enzyme stability; fluorescence emission; inactivation rates; guanidine HCl.

INTRODUCTION

Glutaryl-7-ACA acylase (gl-7-ACA acylase) is an amide hydrolase that specifically catalyzes the cleavage of the glutaryl side chain of glutaryl-7-amino cephalosporanic acid (glutaryl-7-ACA) (Scheme 1) or its desacetoxy derivative, glutaryl-3-desacetoxy-7-amino cephalosporanic acid (glutaryl-7-

*Author to whom all correspondence and reprint requests should be addressed.



Scheme 1

ADCA). The products of the deacylation reactions are the corresponding 7-amino cephalosporanic acids, 7-ACA and 7-ADCA, which are key intermediates for the preparation of semisynthetic cephalosporanic antibiotics *(1)*. In order to overcome the problems associated with the chemical synthetic route (expensive techniques and environmentally risky reactants and byproducts), an enzyme-mediated process for the synthesis of 7-ACA from cephalosporin C has been proposed as an alternative route to conventional industrial chemical processes *(2,3)*. It consists of two consecutive enzymatic steps (Scheme 1): the first step is an oxidative deamination of cephalosporin C by a specific D-amino acid oxidase; the second step is the deacylation of the resulting glutaryl derivative by a specific glutaryl-7-ACA acylase. From an industrial point of view, the relevance of gl-7-ACA acylase is therefore related to its rather selective specificity toward substrates containing the β-lactam ring.

Gl-7-ACA acylase activity was detected in several microorganisms *(4,5)*, whose growth and metabolic pathways were studied to optimize enzymatic production. The sequence of gl-7-ACA acylase gene from *Pseudomonas*, cloned and expressed in a *Escherichia coli* host *(6)*, has been reported *(7)*. Almost all the gl-7-ACA acylases characterized so far are heterodimers derived from a single polypeptide precursor that undergoes a proteolytic asymmetric cleavage after synthesis. The two fragments are then assembled to form the mature protein.

In spite of the relevance as an enzyme of industrial interest, gl-7-ACA acylase has been only marginally characterized from a biochemical point of view *(8)*. Although some biotechnical aspects of the enzymatic process have been studied (i.e., immobilization onto supports and definition of a

bioreactor for the continuous production of 7-ACA) *(9–11)*, the performance of the enzyme has been addressed more specifically than the biochemical properties relevant for applications.

In this study, gl-7-ACA acylase from recombinant *E. coli* strain NCIMB 40560, containing the gl-7-ACA acylase gene of the soil isolate *Pseudomonas sp.* NCIMB 40474 *(12)*, has been purified to homogeneity and partially characterized. The enzyme stability and inactivation kinetics were studied as a function of some chemical and physical parameters (denaturant concentration, pH, and temperature). It was found that the monomer–dimer equilibrium plays an important role in enzymatic activity and stability.

MATERIALS

Ammonium sulfate, sodium phosphate, acetic acid, and 7-ADCA were purchased from Carlo Erba (Italy). Glutaryl-7-ADCA was synthesized as previously described *(5)*. Glutaric acid and *p*-dimethylaminobenzaldeyde were from Fluka (Buchs, Switzerland). Guanidine HCl, Tris, Blue Dextran, and potassium pyrophosphate were from Sigma (Milano, Italy). Anpholine PAG plates for isoelectric focusing were from LKB-Pharmacia (Uppsala, Sweden).

METHODS

Cell Culture

Recombinant *E. coli* strain was cultivated in a fed-batch fermenter as described elsewhere *(12)*. The cellular mass yield was 60 g/L (wet wt) and the activity was 4000–5000 U/L of fermentation broth.

Purification of gl-7-ACA Acylase

50 g of *E. coli* cell paste were suspended in 250 mL of 100 m*M* sodium phosphate buffer, pH 7.0, containing 5 m*M* glutaric acid. The cells were disrupted by ultrasonic treatment (Brown cell homogenizer) for seven cycles of 2 min each at 0°C, with intermediate cooling. The temperature never exceeded 10°C. The suspension was centrifuged at 15000*g* for 20 min, and the sedimented cellular debris were discarded. The pH of the supernatant was changed to pH 11.0 and ammonium sulfate concentration was adjusted to 30% saturation. After 40–60 min, the pH was brought back to pH 8.0 and the suspension centrifuged (15,000*g*, 20 min). The precipitate obtained after addition of ammonium sulfate (75% saturation, overnight), was resuspended in 100 mL of Tris buffer 50 m*M*, pH 8.0, NaCl 50 m*M*, dialyzed, and applied to a DEAE-Sephacel column (33 × 350 mm) equili-

brated with the same buffer. The column was then eluted with a linear gradient of NaCl (50–400 m*M*) (1800 mL, 40 mL/h). The active fractions were pooled, dialyzed against phosphate buffer 10 m*M*, pH 6.5, ammonium sulfate (30% saturation), and applied to a octyl-Sepharose Cl-4B column (22 × 300 mm). The acylase was eluted with a linear gradient of ammonium sulfate (30–0% saturation, 750 mL, 15 mL/h). After dialysis against 2 m*M* phosphate buffer, pH 6.8, fractions containing acylase activity were applied to a hydroxyapatite column (32 × 300 mm). The column was filled (two-thirds) with glass beads (6-mm diameter) and equilibrated with the same buffer. The acylase activity was eluted with a linear sodium phosphate gradient from 100 to 10 m*M* in a total volume of 500 mL. Finally, the fractions containing gl-7-ACA acylase were pooled, dialyzed against phosphate buffer 100 m*M*, pH 7.5, NaCl 50 m*M*, and applied to a column of Sephacryl S-300 (22 × 1000 mm) equilibrated with the same buffer. Active fractions were pooled and stored at –20°C.

Enzymatic Activity Assay

The activity assay of gl-7-ACA acylase was routinely performed by using glutaryl-7-ADCA (gl-7-ADCA) as substrate, according to the method of Balashingam *(13)*, modified as follows: 1 mL of enzyme solution (0.01–0.02 mg/mL) in phosphate buffer 100 m*M*, pH 7.5, was incubated at 37°C for 15–30 min in the presence of gl-7-ADCA (6.5 m*M*). The reaction was stopped with 3 mL of an aqueous solution of acetic acid (7% v/v) and NaOH (0.015 *M*). Then, 1 mL of *p*-dimethylamino-benzaldehyde (PDAB, 0.5% w/v in methanol) was added to the assay mixture. The units (U) of acylase activity were calculated by the following equation: U/mL = $(A_{415})/(0.4xt)$, in which A_{415} is the difference in the absorbance at 415 nm observed at t (reaction time, min), and 0.4 is the extinction coefficient at 415 nm of the yellow-colored Schiff base formed between DAB and the free amino group of 7-ADCA. The substrate concentration ranged from 0.05 to 7 m*M*. One unit of acylase activity was defined as the amount of enzyme that produced 1 µmol of 7-ADCA per min at 37°C, pH 7.5.

The inhibition of glutaric acid on the activity of gl-7-ACA acylase (0.01–0.02 mg/mL) was assayed in phosphate buffer 100 m*M*, pH 7.5, in the presence of different initial concentrations of the acid (from 0 to 0.3% w/v). The same volume of substrate solution at different concentrations was added to the enzyme solution containing a given amount of glutaric acid. The final reaction volume was 2.5 mL. After 10–30 min, the reaction was stopped by adding the regents necessary for the spectrophotometric assay. The initial velocity was calculated as a function of substrate concentration, and the apparent values of k_{cat} and K_M were calculated with the software ENZFITTER (Biosoft, UK), according to Michaelis-Menten

kinetics mechanism. From the curves at different inhibitor concentrations, the apparent inhibition constant, K_I was calculated.

Inactivation in the Presence of Guanidine

Aliquots of gl-7-ACA acylase stock solution (1 mg/mL, in phosphate buffer 100 m*M*, pH 7.0) were added to 1.25 mL of guanidine hydrochloride (GuHCl) dissolved in the same buffer solution at different concentration (up to 2 *M*), with or without β-mercaptoethanol, 5 m*M*. After 1 h at 25°C, the residual activity was assayed by adding glutaryl-7-ADCA (final concentration 6 m*M*). The sample were allowed to react for 15–30 min at 37°C, and then the spectrophotometric activity test was carried out as described in the Enzymatic Activity Assay section. Control experiments confirmed that the Michaelis-Menten kinetic mechanism was not affected by the presence of GuHCl.

Fluorescence Spectroscopy

Photoluminescence spectra were measured at 20°C with a SPEX spectrofluorometer (mod. 2221), with double monochromators both in excitation and emission equipped with a 450 W xenon lamp. The excitation wavelength was 290 nm or 295 nm. Detection was achieved with a photomultiplier tube, operating in single-photon counting, with a thermoelectrically cooled photocatode at 40°C. Spectra were corrected for optics and detector efficiency. Samples were prepared by diluting a concentrated protein stock solution by weight until a final concentration of about 0.05 mg/mL (phosphate buffer 100 m*M*, pH 7.5, with and without GuHCl 6 *M*), in which inner filter effects were absent.

Quantum yields were obtained from the equation: $Q/Q_0 = (I/I_0)(A_0/A)$ *(14)*, in which Q and Q_0 are quantum yields, I and I_0 are areas under the fluorescence peaks, and A and A_0 are the absorbances at 280 nm of the sample and the standard, respectively. Absolute quantum yields of gl-7-ACA acylase (phosphate 100 m*M*, pH 7.0) were calculated by using L-tryptophan as standard ($Q_0 = 0.13$) *(15)*.

Protein Concentration

The concentration of gl-7-ACA acylase was determined with the method of Bradford *(16)*. Bovine serum albumin was used as standard.

Gel Filtration Chromatography

A column (1.6 × 100 cm) packed with Superdex-200 (LKB-Pharmacia) was equilibrated with phosphate buffer 100 m*M*, NaCl 50 m*M*, pH 7.5, at different concentrations of GuHCl, or with acetate buffer 100 m*M*, pH 5.0. The temperature and flow rate were 20 ± 0.1°C and 10 mL/h, respectively.

The protein sample loaded on the column was always 1 mL. The concentration of gl-7-ACA acylase ranged from 1.5 to 0.01 mg/mL; the concentration of the proteins used as mol wt standard was 1 mg/mL. The column was repacked and calibrated at each guanidine concentration. Calibration was performed by using the following proteins as mol wt standards: ribonuclease A (mol wt 13,700), bovine serum albumin (mol wt 66,000), β-galactosidase from *E. coli* (mol wt 116,000), and Blue Dextran 2000. Interpolation of the calibration curve based on the experimental retention coefficient, V_e/V_o where V_e is the elution volume of the standard proteins (the position of the protein peaks in the elution diagram) and V_o is the void volume, allowed the estimate of the sample mol wt *(17)*. Only small changes in the retention coefficients of the proteins used as standards were observed as a function of GuHCl concentration up to 1 *M*.

Dependence of gl-7-ACA Acylase Activity on pH

Aliquots of a protein stock solution were transferred into 2 mL of different buffer solutions. The final enzyme concentration was 0.1 mg/mL. Acetate, phosphate, Tris, and K-pyrophosphate buffers 50 m*M*, NaCl 50 m*M*, were used in the pH ranges 4.5–6.0, 6.0–7.0, 7.0–9.0 and 9.0–10.0, respectively. After a few minutes of temperature equilibration at 37°C, the reaction was started by adding the substrate (glutaryl-7-ADCA, 6 m*M* final concentration). After 15–20 min, 0.5 mL of solution was withdrawn and analyzed spectrophotometrically as described in the Enzymatic Activity Assay section.

Storage Stability

The stability of the gl-7-ACA acylase activity was studied at pH 7.0, pH 6.0 (phosphate buffer 100 m*M*), and pH 5.0 (acetate buffer 100 m*M*) as a function of storage time. Three protein stock solutions (0.1 m/mL) at different pH were incubated at 28°C. Aliquots were withdrawn at different time intervals and assayed to control the residual activity. Glutaryl-7-ADCA was used as substrate. The same procedure was applied to check the enzyme stability at pH 7.0 as a function of the temperature. The time-dependent residual activity was measured at 35, 40, 42, and 45°C, respectively.

Isoelectric Focusing

IEF experiments were performed with a flat-bed unit FBE 3000 (Pharmacia) by using Anpholine PAG plates (polyacrylamide gels precast on polymer support film). The pH range of the gels was 3.5–9.5. Phosphoric acid and sodium hydroxide 1 *M* were used as anode and cathode solutions, respectively. Samples were focused at 30 W (1200 V) for 90 min. A standard mixture of proteins of known isoelectric point was used as reference (pH range: 4.4–9.6, Bio-Rad, Italy). The migration dis-

Table 1
Purification of Recombinant Glutaryl-7 ACA Acylase from 1 L of *E. coli* Culture

Purification step	*Units*[a]	*U/mg*	*yield, %*
Cell lysate	4000	0.10	100
pH 11, ammonium sulfate 30%	3500	0.23	87
Ammonium sulfate 75%	3100	0.6	78
DEAE-Sephacel	1900	1.2	47
Octyl-Sepharose Cl-4B	1610	3.5	40
Hydroxyapatite	800	4.15	20
Sephacryl S300	700	4.41	17

[a] glutaryl-7-ADCA as substrate, pH 7.5, 37°C.

tance from the cathodic edge of the gel of the standards plotted vs the corresponding p*I* was used to calculate the calibration curve.

RESULTS

Purification, Activity, and Inhibition Studies

The procedure for the gl-7-ACA acylase purification from crude cell extracts is summarized in Table 1. As judged by scan densitometry of SDS-disc-electrophoretic gels, the purity of the gl-7-ACA acylase preparation was higher than 95%. Moreover, if the specific activity of the crystalline glutaryl acylase from *Pseudomonas* SY strain (4.66 U/mg) *(18)* is used as reference, again the purity of gl-7-ACA acylase (4.41 U/mg, *see* Table 1) is about 95%. Two bands were detected by standard SDS DISC electrophoresis, corresponding to a mol wt of 18 and 52 kDa. Since the mol wt of intact protein measured by gel filtration chromatography is 134 kDa, the enzyme is probably a tetramer of the type $\alpha_2\beta_2$, composed of a couple of asymmetric dimers. The mol wt of the two subunits are slightly different from those reported for the two subunits (16 and 54 kDa) of gl-7-ACA acylase from *Pseudomonas* SY *(19)*. Isoelectric focusing (IEF) experiments showed the presence of two bands corresponding to a p*I* of 4.9 and 4.4, respectively, probably associated with the two subunits. The value is similar to that of gl-7-ACA acylase from *Bacillus laterosporus* (p*I* 4.7) *(20)*.

The observed values of k_{cat} and K_M for the hydrolysis of glutaryl-7-ACA at 37°C, pH 7.5, calculated according to Michaelis-Menten kinetic mechanism, are 37 s^{-1} and 0.37 $mmol^{-1}$, respectively. Both parameters are in good agreement with the literature values *(18)*.

When the concentration of the substrate was increased (up to 1–2% w/v), it was noticed that the substrate conversion was not linear as a

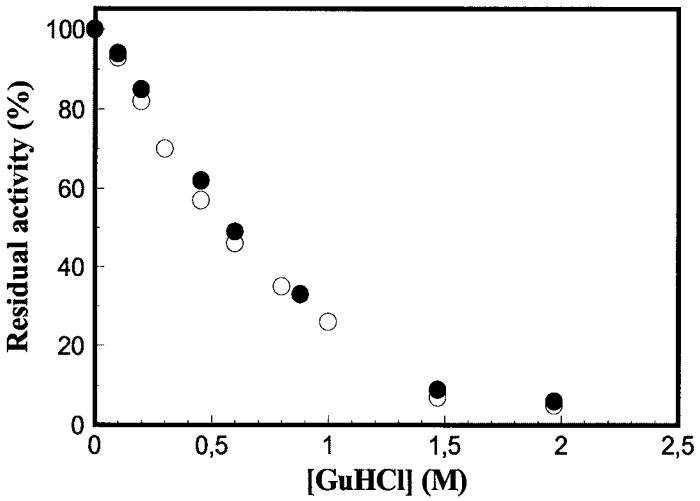


Fig. 1. Inactivation of the glutaryl-7-ACA acylase activity as a function of guanidine HCl. Residual activity of glutaryl-7-ACA acylase (substrate: gutaryl-7-ADCA, 6m*M*) incubated for 60 min, 25°C, in phosphate buffer 100 m*M*, pH 7.0 as a function of guanidine HCl, with (○) or without (●) β-mercaptoethanol (5 m*M*).

function of time. This effect was caused by the progressive inhibition by glutaric acid released by cleavage of the glutaryl side chain. Double reciprocal plots (Lineweaver-Burk plots) at different inhibitor concentrations showed that the acid is a competitive inhibitor. The apparent inhibition constant, K_I, of the reaction is 0.16 ± 0.03 m*M*.

Inactivation by GuHCl

In Fig. 1, the residual activity of gl-7-ACA acylase is plotted as a function of GuHCl concentration. As can be seen, the sensitivity of the enzyme toward GuHCl is quite high: The residual activity steadily decreases, starting from concentrations as low as 0.1 *M*. The effect of GuHCl cannot be attenuated by the presence of a mild reducing agent such as mercaptoethanol (Fig. 1, empty circles). This implies that the loss of activity does not necessarily include the oxidation (reduction) of some key functional protein groups.

The reversibility of the gl-7-ACA acylase inactivation or unfolding was tested after exposure to GuHCl 4 *M* for 30 min at 25°C. The sample was then diluted 300 times in phosphate buffer 100 m*M*, pH 7.0 and the recovery of the protein activity was followed as a function of time. No sign of activity was observed after several hours (up to 20), suggesting that the enzyme was irreversibly inactivated (denaturated) after exposure to GuHCl 4 *M*.

The fluorescence spectra of gl-7-ACA acylase were recorded as a function of GuHCl concentration by using the excitation wavelength at 290 nm, when only trytophan residues are excited *(21)*. The results are

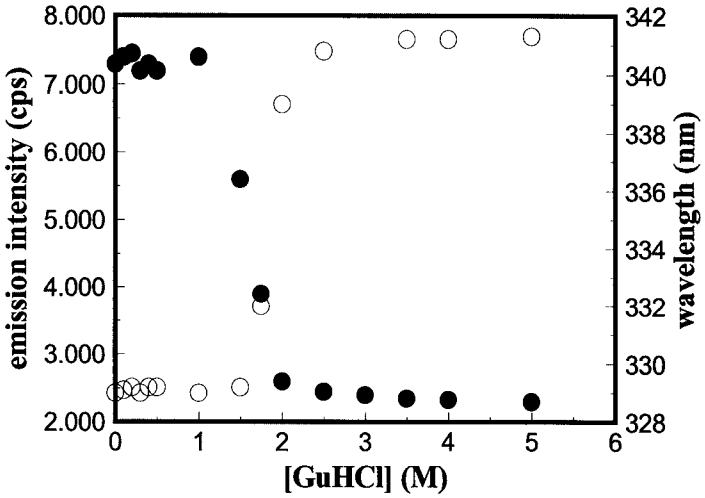


Fig. 2. Fluorescence intensity of glutaryl-7-ACA acylase as a function of guanidine HCL. Fluorescence emission intensity (●) and maximum intensity position (○) of glutaryl-7-ACA acylase in phosphate buffer 100 m*M*, pH 7.0, 20°C as a function of GuHCl concentration. Fluorescence intensity was normalized with respect to the protein concentration (0.03 mg/mL).

shown in Fig. 2, in which the fluorescence intensity and the wavelength shift of the emission peak are plotted as a function of the denaturant concentration. Both curves undergo a sharp change around 1.8 *M*. Are the fluorescence spectral changes related to the unfolding of the protein structure? Control experiments showed that the spectrum of the protein tryptophan residues at high GuHCl concentration almost overlaps that of the free amino acid in solution, suggesting that the protein in these conditions is in the unfolded state.

The calculated tryptophan quantum yields were 0.134 and 0.08 for the native and denatured forms, respectively. They are close to the values typical of other proteins *(15)*. The decrease in quantum yield after unfolding is common to some proteins *(22)*, in accordance with changes of the quenching effects after exposure of the chromophores (or most of them) to the solvent. The position of λ_{max} of the native state spectrum at 331.5 nm is typical of the class of proteins whose tryptophan residues are located in the inner core of the protein interior and shielded from the bulk solvent *(23)*.

From the analysis of the λ_{max} shift as a function of GuHCl concentration on the assumption that the transition follows a two-state behavior, the apparent free energy of unfolding, ΔG_0, can be calculated by extrapolation to zero GuHCl concentration *(20)*. By plotting ΔG vs denaturant concentration, a straight line is obtained (R = 0.988), with intercept at 0 *M* GuHCl, ΔG_0, of 33 ± 3 KJ/mol and slope, *m*, of 17 KJ/mol/*M*. The value of ΔG_0 falls in the range of a stable globular protein *(25)*.

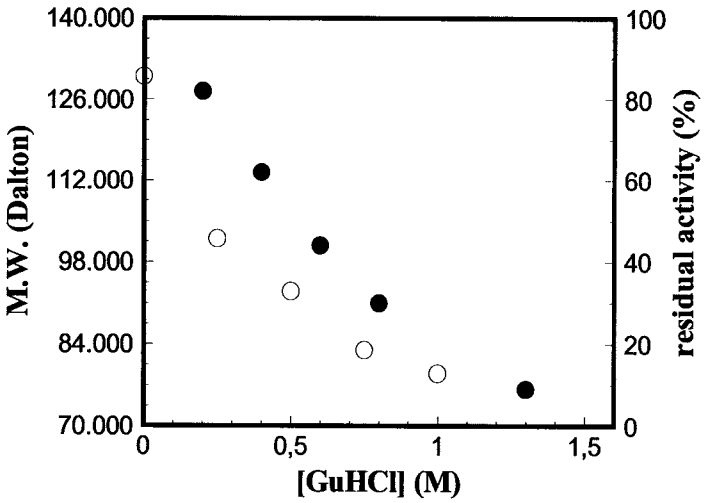


Fig. 3. Apparent mol wt of glutaryl-7-ACA acylase as function of guanidine HCl. The mol wt were obtained by gel filtration chromatography with a column (1.6 × 100 cm) of Superdex 200 in phosphate buffer 100 m*M*, NaCl 50 m*M*, pH 7.5, 20°C. Sample volume: 1 mL. Protein concentration: 1 mg/mL. (○) apparent mol wt, (●) residual activity (same as in Fig. 1).

Molecular Weight Analysis

Because protein unfolding takes place at GuHCl concentrations above 1.9 *M* (as suggested by the analysis of the spectroscopic measurements), one may ask which is the origin of the inactivation process observed at lower denaturant concentration. In order to answer this question, the apparent mol wt of gl-7-ACA acylase has been studied as a function of GuHCl concentration by gel filtration chromatography. The results are presented in Fig. 3. As can be seen, the apparent mol wt of gl-7-ACA acylase decreases steadily from about 140 to 79 kDa as the concentration of GuHCl is increased from 0 to 1 *M*, suggesting that the tetrameric enzyme dissociates into dimers in that GuHCl concentration range. The trend of the molecular size as a function of GuHCl concentration is quite similar to the decay of the enzyme activity (Fig. 3, filled circles).

In the absence of GuHCl at neutral pH, the apparent mol wt of gl-7-ACA acylase, obtained by gel-filtration chromatography in the concentration range of 1.–1.5 mg/mL, was 134 kDa, a value close to the theoretical mol wt of the tetramer, calculated from SDS-electrophoresis.

pH Studies

The dependence of the gl-7-ACA acylase activity on pH is shown in Fig. 4. The activity is almost pH-independent between pH 6.0 and pH 10.0, but it decreases sharply below pH 6.0. In order to verify whether changes

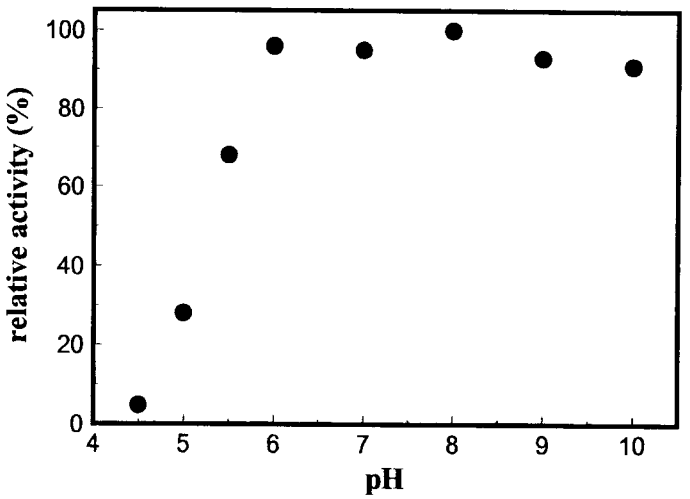


Fig. 4. pH dependence of the glutaryl-7-ACA activity. Acetate, phosphate, Tris, and potassium pyrophosphate buffers 50 m*M*, NaCl 50 m*M* were used in the pH ranges 4.5–6.0, 6.0–7.0, 7.0–9.0, and 9.0–10.0, respectively.

in the subunit composition occurred below pH 6.0, the apparent mol wt of the protein was studied at pH 5.0 by gel filtration chromatography. The observed apparent mol wt was 88.1 ± 2 kDa, suggesting a significant degree of dissociation of the enzyme. It is interesting to note that the protein sample eluted from the column and dialyzed against phosphate buffer 100 m*M*, pH 7.0 was 95% as active as the starting enzyme. Thus, it seems that dimer–tetramer equilibrium is reversible at pH 5.0.

Stability as a Function of pH and Temperature

The stability of gl-7-ACA acylase activity on storage was studied at different pHs, as shown in Fig. 5. At neutral pH, 28°C, the residual activity after 10 d was 95% of the initial one; at lower pHs, the stability progressively worsened. In Fig. 6, the residual activity is plotted as a function of the incubation time at different temperatures. As can be seen, inactivation becomes relevant above 40°C, and over a narrow temperature interval.

The apparent rate constants of the inactivation process, k, were calculated by a single-exponential best fitting of the data shown in Figs. 5 and 6. The equation used was $A = A_0 + A_1e^{-kt}$, where A and A_0 are the residual activity at time t and infinite time, respectively, and k the rate constant. The results are summarized in Table 2.

DISCUSSION

The unfolding of the protein is a highly cooperative process, reflected by the sharpness of the transition as a function of GuHCl. However, the changes

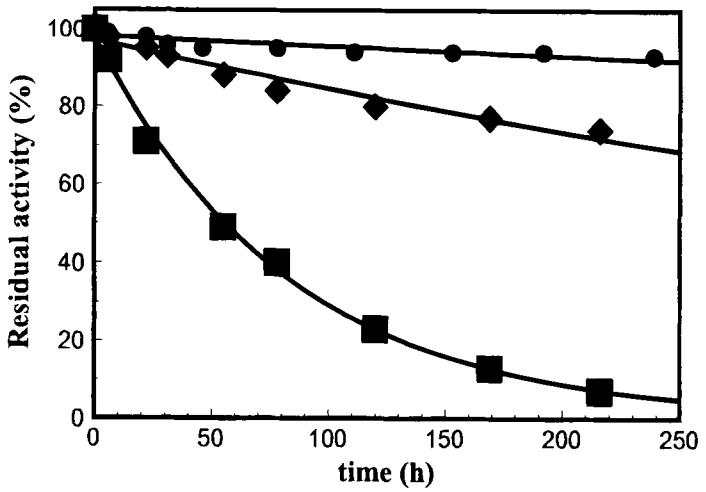


Fig. 5. Long-term stability of glutaryl 7-ACA acylase: temperature dependence. Residual activity as a function of storage time. The enzyme solutions were incubated at 28°C. (●) phosphate buffer 100 m*M*, pH 7.0; (◆) phosphate buffer 100 m*M*, pH 6.0; (■) acetate buffer 100 m*M*, pH 5.0. Lines are exponential best fittings of the data.

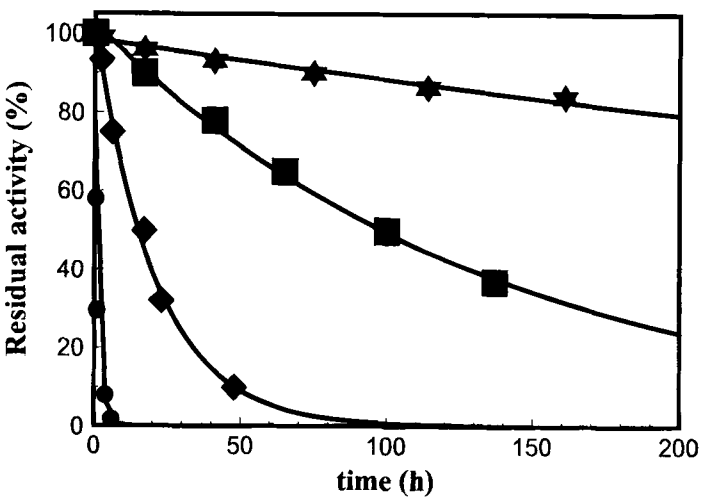


Fig. 6. Long-term storage stability of glutaryl 7-ACA acylase: pH dependence. Residual activity in phosphate buffer 100 m*M*, pH 7.0, at 35°C (★), 40°C (■), 42°C (◆) and 45°C (●). Lines are exponential best fittings of the data.

in the subunit interactions and inactivation are not concomitant to the unfolding process. In fact, activity is lost in a concentration range of GuHCl significantly lower than that at which denaturation occurs. At 1.5 *M*, more than 90% of the activity is already lost (Fig. 1), but the protein conformation, as monitored by fluorescence, is still preserved (Fig. 2). The change of the apparent mol wt (Fig. 3) follows the inactivation dependence on

Table 2
Apparent Inactivation Rate Constants, k, at Different Temperature and pH Values

pH	temperature (°C)	k (h^{-1})
7	28	–
6	28	0.08 (±0.004)[a]
5	28	0.013
7	35	0.0012
7	40	0.0065 (±0.0004)[a]
7	42	0.052
7	45	1.2 (±0.1)[a]

[a]three replicates, standard deviation in the parenthesis.

GuHCl concentration, thus suggesting that the process of inactivation is related to the change of the association state of the protein, rather than to unfolding. More precisely, at 0.8–1 *M* GuHCl, the apparent mol wt is 81 kDa, close to that of the dimer, and the activity is almost 20% of the initial one. This implies that the enzymatically active species is the tetramer and that the dimer is inactive or has a significantly lower activity. The behavior of the gl-7-ACA acylase properties as a function of denaturant concentration is different from that of the closely related penicillin acylase, another enzyme specific for substrates containing β-lactam rings. Unfolding and complete inactivation are concomitant to the dissociation of the dimer into monomers *(26)*.

In order to verify whether the protein dissociation is pH dependent, the apparent mol wt was determined at pH 5.0 by gel chromatography. The observed mol wt, 88 kDa, is close to that of the dimer. At pH 5.0, the change of the mol wt is 75% of that expected for the tetramer to dimer total conversion. This roughly corresponds to the activity loss (72%) at pH 5.0 with respect to pH 6.0 (Fig. 4). It should be noted that the inactivation at acid pH is almost fully reversible (*see* Results). Conversely, recovery of the activity (and consequently, refolding of the protein structure) was not observed after exposure to 4–6 *M* GuHCl.

The pH dependence of the gl-7-ACA acylase long-term storage stability (Fig. 5) may be explained by considering a molecular mechanism similar to that observed for penicillin acylase. The larger subunit of the protein, once isolated from the smaller one, i.e., when the dimer is dissociated into subunits, is not stable in aqueous solution, but tends to aggregate irre-

versibly, making refolding or renaturation impossible *(26)*. Because below pH 6.0 gl-7-ACA acylase dissociates more easily, it is possible that the decrease of the storage stability at acid pH is related to the lower stability of the dimer with respect to the tetramer.

Although the observed inhibition constant, K_I, (0.16 m*M*) is not very low, nevertheless inhibition reactions may cause significant problems when quantitative conversions and high yields are required in large-scale processes. Moreover, the presence of glutaric acid, besides the inhibitory effects, tends to lower the pH of the reaction medium with further inactivation of the enzyme, if the pH decreases below pH 6.0. Consequently, process lines for the production of 7-ACA should be engineered in such a way to continuously remove glutaric acid from the reaction in order to keep its concentration as low as possible (0.5% w/v already means 10% enzyme activity inhibition).

The general picture obtained from the biochemical characterization of the recombinant gl-7-ACA acylase from *Pseudomonas* NCIMB 40474 is that of a relatively stable protein whose activity is sensitive to the oligomeric state of the molecule. It is resistant to appreciable concentration of GuHCl, since its unfolding transition occurs over a narrow range of denaturant concentration, with a midpoint at 1.91 *M* GuHCl. However, inactivation rates become significant already at 42°C and below pH 6.0 (Table 2).

Finally, the lability of the tetramer–dimer subunit interactions may raise problems when the enzyme has to be used as biocatalyst. In order to improve stability, either in operating or storage conditions, several methods may be considered, such as cosolute or cosolvent mixtures, as well as immobilization techniques. In this regard, for instance, a glutaryl-7-ACA acylase from *Pseudomonas* was immobilized on controlled pore glass beads through glutaraldehyde-mediated coupling *(11)*. Although the potentiality of the enzyme as catalyst was significantly improved, the expressed activity and stability after several catalytic cycles was not optimal. If this is related to the dissociation of the tetrameric active form into inactive dimers, improved immobilization conditions have to be specifically envisaged. These may include, as in the case of penicillin-G-acylase, multipoint attachment to the support *(27)* or *ad hoc* support modification *(28)*.

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