

# Catalytic properties of an endogenous $\beta$ -lactamase responsible for the resistance of *Azospirillum lipoferum* to $\beta$ -lactam antibiotics

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*Azospirillum lipoferum* RG20, a nitrogen-fixing bacterium found in all kind of soils, was found to be naturally resistant to penicillins and cephalosporins. 6- $\beta$ -Bromopenicillanic acid, an irreversible inhibitor of serine- $\beta$ -lactamases, completely abolished this resistance. A  $\beta$ -lactamase was purified 518-fold from a cell-free extract of *A. lipoferum* RG20. A single band on SDS-PAGE (apparent molecular mass 31 000 Da) and on isoelectric focussing (pI 9.35) was observed with the purified protein. The enzyme hydrolysed benzylpenicillin, ampicillin, cephalothin and cephaloridine with comparable  $k_{\text{cat}}$  values and catalytic efficiencies. However, carbenicillin and cefotaxime were hydrolysed with significantly lower kinetic parameters and oxacillin was hydrolysed at a rate 100 times slower. The purified  $\beta$ -lactamase was inhibited by clavulanic acid and sulbactam but not by EDTA or aztreonam. Its substrate and inhibitor profiles are consistent with those of the broad-spectrum  $\beta$ -lactamases inhibited by clavulanic acid (group 2b of the Bush–Jacoby–Medeiros scheme). The effect of pH on  $k_{\text{cat}}$  and  $K_{\text{m}}$  values for benzylpenicillin hydrolysis was studied. The dependence of  $k_{\text{cat}}$  on pH suggests that the enzyme–substrate (ES) complex must be in at least three protonation states: two with  $k_{\text{cat}}$  values equal to 2800 and 1450 s<sup>-1</sup> and a third inactive one [ $\text{p}K_{1(\text{ES})}$  4.7 and  $\text{p}K_{2(\text{ES})}$  7.9]. Similarly, the dependence of  $k_{\text{cat}}/K_{\text{m}}$  on pH can be explained by postulating that the enzyme free form can be at least in three different protonation states: two of them with  $k_{\text{cat}}/K_{\text{m}}$  values equal to  $2.7 \times 10^6$  and  $3.7 \times 10^8$  M<sup>-1</sup> s<sup>-1</sup> and a third one unable to productively bind substrate. Interestingly, the dependence of  $k_{\text{cat}}/K_{\text{m}}$  on pH is consistent with positive cooperativity for proton binding to the enzyme free form [ $\text{p}K_{1(\text{E})}$  8.5 and  $\text{p}K_{2(\text{E})}$  7.2].

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## INTRODUCTION

The production of  $\beta$ -lactamases (EC 3.5.2.6) is the most prevalent mechanism of bacterial resistance to  $\beta$ -lactam antibiotics (Philippon *et al.*, 1998). These enzymes were detected in bacteria before the widespread therapeutic use of penicillin (Abraham & Chain, 1940) and their presence in many pathogenic and non-pathogenic micro-organisms – including phototrophic bacteria – has been reported (Baumann *et al.*, 1989; Bush *et al.*, 1995). It has been proposed that  $\beta$ -lactamases are evolutionarily related to cell-wall biosynthetic enzymes, viz. the penicillin-binding proteins. It is presumed that some micro-organisms evolved  $\beta$ -lactamases in order to overcome the challenge of  $\beta$ -lactam antibiotics, which were being exuded in the environment by their competitors (Massova & Mobashery, 1998).

*Azospirillum* spp. are widely distributed soil nitrogen-fixing

bacteria that live in close proximity to plant roots, and they play an important role in the promotion of plant growth (Steenhoudt & Vanderleyden, 2000). Wild-type strains of *Azospirillum lipoferum* and *Azospirillum brasilense* were found to be naturally resistant to penicillins, a resistance that was attributed to  $\beta$ -lactamase activity (Franche & Elmerich, 1981). We have previously reported conclusive evidence for the presence of an inducible  $\beta$ -lactamase in *A. lipoferum* RG20 (Boggio *et al.*, 1989). In this study, we provide experimental evidence demonstrating that this enzyme is responsible for the natural resistance of *A. lipoferum* RG20 to  $\beta$ -lactam antibiotics. We also report the purification and catalytic properties (substrate, inhibitor and pH profiles) of the benzylpenicillin-induced *A. lipoferum* RG20  $\beta$ -lactamase.

## METHODS

**Bacterial strain.** *A. lipoferum* RG20 (BR11115) was isolated by Embrapa Agrobiologia (Empresa Brasileira de Pesquisa Agropecuária, Brasil).

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**Susceptibility tests.** MIC values were determined in test tubes filled with 3 ml of Luria–Bertani (LB) medium (Ausubel *et al.*, 1993), which were inoculated with 0.1 ml of an exponential-phase culture ( $5 \times 10^7$  cells  $\text{ml}^{-1}$ ). The antibiotics were added at various concentrations and the cells were incubated for 18 h at 37 °C.

**Induction tests.** The  $\beta$ -lactam compounds to be tested ( $100 \mu\text{g ml}^{-1}$ ) were added to a culture in exponential phase ( $\text{OD}_{540}$  value of between 0.8 and 0.9). Cells were harvested 1 h later by centrifugation at 4000 g for 10 min at 4 °C and washed with 100 mM potassium phosphate buffer (pH 7.0). The suspension of washed cells was immersed in an ice–water slurry bath and sonicated (two 30 s pulses) with an MSE 150 Ultrasonic Desintegrator. The sonicate was centrifuged at 12 000 g for 15 min at 4 °C to remove cells debris.  $\beta$ -Lactamase activity, expressed as mU per  $10^{-9}$  cells, was determined spectrophotometrically in the supernatant using nitrocefin ( $\Delta\epsilon_{482} = 15\,900 \text{ M}^{-1} \text{ cm}^{-1}$ ) as substrate (O’Callaghan *et al.*, 1972). The activity determined in extracts from cells grown in the absence of antibiotic was 20 mU per  $10^{-9}$  cells.

**Enzyme assays.**  $\beta$ -Lactamase activity was measured spectrophotometrically in 1 ml quartz cuvettes at 30 °C (Waley, 1974). The differential absorption coefficients (unhydrolysed minus hydrolysed) for the different antibiotics used were: benzylpenicillin,  $\Delta\epsilon_{230} = -1090 \text{ M}^{-1} \text{ cm}^{-1}$ ; ampicillin,  $\Delta\epsilon_{233} = -780 \text{ M}^{-1} \text{ cm}^{-1}$ ; carbenicillin,  $\Delta\epsilon_{232} = -700 \text{ M}^{-1} \text{ cm}^{-1}$ ; cephaloridine,  $\Delta\epsilon_{260} = -9490 \text{ M}^{-1} \text{ cm}^{-1}$ ; cefotaxime,  $\Delta\epsilon_{262} = -5990 \text{ M}^{-1} \text{ cm}^{-1}$ ; cephalothin,  $\Delta\epsilon_{263} = -7380 \text{ M}^{-1} \text{ cm}^{-1}$ ; oxacillin,  $\Delta\epsilon_{263} = 290 \text{ M}^{-1} \text{ cm}^{-1}$ . The reaction was started by the addition of the enzyme to a medium containing 100 mM sodium phosphate buffer (pH 7.0) and appropriate concentrations of the antibiotics. One enzyme unit (U) is defined as the amount of enzyme that hydrolysed 1  $\mu\text{mol}$  of substrate in 1 min under assay conditions.

**Purification of the enzyme.** *A. lipoferum* RG20 was grown in 6 l Erlenmeyer flasks containing 1.5 l of LB medium in a New Brunswick Giratory Water Bath Shaker (250 r.p.m.) at 37 °C. Benzylpenicillin ( $100 \mu\text{g ml}^{-1}$ ) was added when the  $\text{OD}_{540}$  value reached between 0.8 and 0.9. One hour later the cells were harvested by centrifugation at 4000 g for 10 min at 4 °C and washed with 100 mM potassium phosphate buffer (pH 7.0). All further operations were carried out at 4 °C. Washed cells were resuspended in 20% sucrose and 30 mM Tris/HCl (pH 7.0), treated with lysozyme as described (Lindström *et al.*, 1970) and centrifuged at 16 000 g for 15 min. The pellet was resuspended in cold water and the suspension was centrifuged at 27 000 g for 30 min. The supernatant (crude extract) was precipitated with ammonium sulphate. Most of the activity was recovered in the 40–85%  $(\text{NH}_4)_2\text{SO}_4$  saturation fraction, which was resuspended in 10 mM potassium phosphate buffer (pH 7.0), dialysed against the same buffer containing 15% (v/v) glycerol (buffer A), and applied to a CM-Sephadex column (1.8 × 16 cm) equilibrated with buffer A. The column was washed with buffer A plus 50 mM NaCl. A 50–500 mM linear NaCl gradient was applied and  $\beta$ -lactamase activity was recovered in the fractions corresponding to about 200 mM NaCl. The more-active fractions were combined, dialysed against 20 mM Tris/HCl buffer (pH 7.0) and 15% (v/v) glycerol (buffer B) and applied to a Cibacron Blue 3GA-Agarose column (1.5 × 10 cm) equilibrated with the same buffer. The column was washed with buffer B plus 100 mM NaCl and subsequently developed with a 100–800 mM linear gradient of NaCl.  $\beta$ -Lactamase eluted at about 200 mM NaCl. The purified enzyme was concentrated using Centriprep 10 concentrators (Amicon) and stored at –20 °C in 20 mM Tris/HCl buffer (pH 7.0) containing 15% (v/v) glycerol.

Enzyme activity was monitored during purification as indicated in the ‘Enzyme assays’ section of Methods using 1 mM benzylpenicillin as substrate.

**Electrophoresis.** SDS-PAGE was performed in 12.5% vertical slab mini-gels by the method of Laemmli (1970). Gels were stained with Coomassie brilliant blue R250.

**Isoelectric focussing.** Analytical isoelectric focussing was performed in a 111 Mini IEF Cell apparatus (Bio-Rad) on polyacrylamide gels in the presence of ampholines in the pH range 8–10.5. Broad-range pI markers from Pharmacia were used. Gels were stained with Coomassie brilliant blue R250. The  $\beta$ -lactamase band was identified by placing filter paper soaked in a 100  $\mu\text{M}$  solution of nitrocefin on the gel (Matthew *et al.*, 1975).

**Molecular mass determination.** The apparent molecular mass of the denatured purified  $\beta$ -lactamase was determined from the mobility of the protein on SDS-PAGE. Molecular mass markers were BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and  $\alpha$ -lactalbumin (14.4 kDa) (Pharmacia).

**Estimation of kinetic parameters.** Initial velocities at different substrate concentrations were determined in duplicate as indicated in the ‘Enzyme assays’ section of Methods.  $K_m$  and  $V_{max}$  values were estimated by fitting the experimentally determined values to the Michaelis–Menten equation using a non-linear regression procedure. An apparent molecular mass of 31 000 Da was used to calculate  $k_{cat}$  values.

**Inhibitor studies.** Initial velocities were determined at different fixed benzylpenicillin concentrations and variable inhibitor concentrations. The reaction was started by addition of the enzyme and was continuously monitored over 1 min as indicated in the ‘Enzyme assays’ section of Methods.  $v_0/v_i$  Values (where  $v_0$  is the initial velocity in the absence and  $v_i$  is the initial velocity in the presence of inhibitor) were plotted against inhibitor concentration  $[I]$  and analysed as described by Roveri (1985). Linear inhibitors yield linear plots whose slopes depend on substrate concentration in a characteristic manner that makes possible the diagnosis of the inhibitor type and the estimation of  $K_i$  values.

**General.** Protein determinations were carried out according to Lowry *et al.* (1951) using BSA as the standard, the concentration of which was determined spectrophotometrically ( $A_{279} = 6.67 \text{ cm}^{-1}$  for a 1% solution; Foster & Serman, 1956).

Concentration of cells was determined by measuring the optical density of a cell suspension at 540 nm ( $\text{OD}_{540} = 2$  for  $10^9$  cells  $\text{ml}^{-1}$ ).

**Chemicals.** Nitrocefin was a generous gift from Glaxo. Antibiotics and  $\beta$ -lactamase inhibitors were obtained as follows: benzylpenicillin, ampicillin, oxacillin, cephalothin, cloxacillin, cephalosporin C and cephaloridine, Sigma; carbenicillin and sulbactam, Pfizer; clavulanic acid and ceftazidime, Smith Kline Beecham; cefotaxime, Aventis; aztreonam, Merck Sharp Dohme. 6- $\beta$ -Bromopenicillanic acid was kindly synthesized, according to Pratt & Loosemore (1978), by Dr O. A. Mascaretti (IQUIOS, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Argentina). Lysozyme and Cibacron Blue 3GA-Agarose were from Sigma. Other chemicals were of analytical grade.

## RESULTS AND DISCUSSION

### Resistance to $\beta$ -lactam antibiotics

*A. lipoferum* RG20 showed high resistance to benzylpenicillin, ampicillin, carbenicillin, cephalosporin C, cephaloridine, cephalothin and cefotaxime ( $\text{MIC} \geq 1000 \mu\text{g ml}^{-1}$ ), whereas it was more susceptible to oxacillin and cloxacillin ( $\text{MIC} = 200 \mu\text{g ml}^{-1}$ ). The ability of these antibiotics to

induce  $\beta$ -lactamase activity was tested. The activity was increased 100- to 300-fold by the antibiotics with higher MIC values and 20-fold by the antibiotics with lower MIC values.

Crude extracts obtained from benzylpenicillin-induced *A. lipoferum* cells were analysed by isoelectric focussing. A single intense band that focussed at pH 9.3 was detected when the gel was specifically stained with nitrocefin as indicated in Methods.

6- $\beta$ -Bromopenicillanic acid, a known irreversible inhibitor of serine  $\beta$ -lactamases (Pratt & Loosemore, 1978), abolished the resistance of *A. lipoferum* to  $\beta$ -lactam antibiotics. 6- $\beta$ -Bromopenicillanic acid ( $0.5 \mu\text{g ml}^{-1}$ ) strongly decreased MIC values for benzylpenicillin (from  $>1000$  to  $0.8 \mu\text{g ml}^{-1}$ ), cephalosporin C (from  $>1000$  to  $0.4 \mu\text{g ml}^{-1}$ ) and oxacillin (from 200 to  $0.4 \mu\text{g ml}^{-1}$ ). These results suggest that *A. lipoferum* resistance to  $\beta$ -lactam antibiotics is mainly due to its inducible  $\beta$ -lactamase.

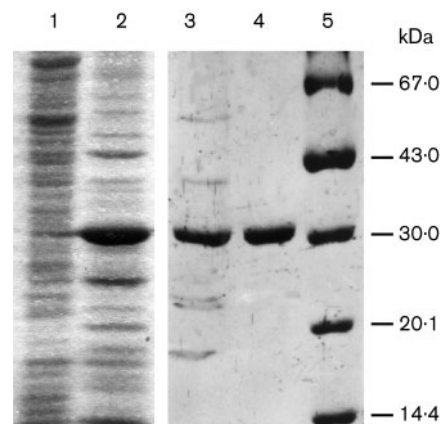
### Purification of $\beta$ -lactamase

$\beta$ -Lactamase from *A. lipoferum* cells induced by benzylpenicillin was purified as indicated in Methods. A final purification of 518-fold was achieved (Table 1). The purified enzyme had a specific activity of  $2155 \text{ U mg}^{-1}$  and was homogeneous on SDS-PAGE and isoelectric focussing.

The position of the single protein band obtained on SDS-PAGE corresponded to a molecular mass of 31 kDa (Fig. 1). Since a value not significantly different (27.5 kDa) was obtained when the apparent molecular mass of the native protein was determined by Sephadex G-100 gel filtration it can be concluded that the  $\beta$ -lactamase of *A. lipoferum* is a monomeric enzyme. A pI value of 9.35 was determined for the enzyme by isoelectric focussing.

### Functional classification

Bush and colleagues have suggested a classification scheme for  $\beta$ -lactamases based on their substrate and inhibitor profiles (Bush, 1989; Bush *et al.*, 1995). This scheme distinguishes four functional groups of  $\beta$ -lactamases. In order to include the *A. lipoferum*  $\beta$ -lactamase in one of these functional groups, we determined the  $\text{Zn}^{2+}$  requirement of the enzyme, the effect of inhibitors such as EDTA, clavulanic



**Fig. 1.** SDS-PAGE for the purification of the  $\beta$ -lactamase from *A. lipoferum* RG20. Lanes: 1, crude extract; 2, ammonium sulphate precipitate; 3, CM-Sephadex eluate; 4, affinity chromatography eluate; 5, molecular mass markers (see Methods).

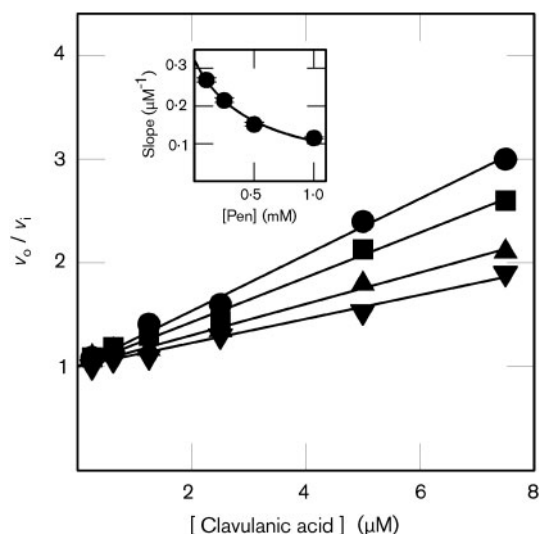
acid, sulbactam and aztreonam, and the kinetic parameters for several  $\beta$ -lactam antibiotics.

The activity of the enzyme was affected neither by exhaustive dialysis against 100 mM potassium phosphate buffer (pH 7.0), 15% (v/v) glycerol and 100 mM EDTA nor by the addition of  $0.25 \text{ mM ZnSO}_4$  to the reaction medium. However, the initial velocity of benzylpenicillin hydrolysis catalysed by the  $\beta$ -lactamase of *A. lipoferum* was inhibited by clavulanic acid. The inhibition was competitive ( $K_i = 3.1 \pm 0.2 \mu\text{M}$ ) with respect to benzylpenicillin (Fig. 2). Sulbactam also behaved as a linear competitive inhibitor with a  $K_i$  value of  $46 \pm 9 \mu\text{M}$  (data not shown). Therefore, the  $\beta$ -lactamase of *A. lipoferum* does not belong to the group 3 metalloenzymes of the Bush–Jacoby–Medeiros scheme because it was not inhibited by EDTA and did not require  $\text{Zn}^{2+}$ . More likely, it is an active-site serine enzyme that can be included in group 2 of the scheme since it was inhibited by clavulanic acid and sulbactam – known active-site-directed  $\beta$ -lactamase inhibitors.

Various penicillins and cephalosporins were hydrolysed by the purified  $\beta$ -lactamase with the  $K_m$  and  $k_{cat}$  values shown in Table 2. The enzyme behaved as a broad-spectrum  $\beta$ -lactamase, since it hydrolysed benzylpenicillin, ampicillin, cephalothin and cephaloridine with comparable  $k_{cat}$  values. Carbenicillin was hydrolysed with a  $k_{cat}$  value 10 times

**Table 1.** Purification of the  $\beta$ -lactamase from *A. lipoferum* RG20

Step	Protein (mg)	Specific activity ( $\text{U mg}^{-1}$ )	Total activity (U)	Purification (-fold)	Yield (%)
Crude extract	837	4	3349	1	100
Ammonium sulphate precipitation	401	8	3208	2	96
CM-Sephadex	1.5	1251	1847	301	55
Affinity chromatography	0.6	2155	1373	518	41



**Fig. 2.** Inhibition of the  $\beta$ -lactamase from *A. lipoferum* by clavulanic acid.  $\beta$ -Lactamase activity was determined as described in Methods at variable benzylpenicillin (Pen) concentrations in the absence ( $v_0$ ) and in the presence ( $v_1$ ) of clavulanic acid.  $v_0/v_1$  Values are plotted against the concentration of clavulanic acid, as described by Roveri (1985). Pen concentrations used were 0.1 mM (●), 0.25 mM (■), 0.5 mM (▲) and 1 mM (▼). Slope values, estimated from the primary plots by linear regression, are plotted in the inset as a function of [Pen]. The line in the inset is the best fit obtained by non-linear regression analysis using the equation  $\text{slope} = K_{\text{Pen}} / \{K_i(K_{\text{Pen}} + [\text{Pen}])\}$ , which describes the behaviour of a linear competitive inhibitor ( $K_i = 3.1 \pm 0.2 \mu\text{M}$ ;  $K_{\text{Pen}} = 0.50 \pm 0.07 \text{ mM}$ ).

**Table 2.** Kinetic parameters of the  $\beta$ -lactamase from *A. lipoferum* RG20

Substrate	$K_m$ ( $\mu\text{M}$ )	$k_{\text{cat}}$ ( $\text{s}^{-1}$ )	$k_{\text{cat}}/K_m$ ( $\mu\text{M}^{-1} \text{ s}^{-1}$ )
Benzylpenicillin	244 ± 11	1321 ± 29	5.4 ± 0.3
Ampicillin	162 ± 9	976 ± 43	6.0 ± 0.4
Cephalothin	342 ± 42	1432 ± 136	4.2 ± 0.7
Cephaloridine	856 ± 95	1137 ± 115	1.3 ± 0.2
Carbenicillin	53 ± 3	129 ± 3	2.4 ± 0.2
Cefotaxime	2918 ± 464	609 ± 95	0.2 ± 0.05
Nitrocefin	34 ± 3	239 ± 14	7.0 ± 0.7

smaller than that of the aforementioned antibiotics. Oxacillin was hydrolysed at a rate 100 times slower than benzylpenicillin. Extended-spectrum antibiotics were not good substrates or ligands for the enzyme: (i) cefotaxime was hydrolysed with catalytic efficiency < 5% that estimated for benzylpenicillin; (ii) aztreonam was not hydrolysed at an appreciable rate by the enzyme; and (iii) 1 mM aztreonam did not inhibit nitrocefin hydrolysis. On these grounds, the enzyme can be allocated to group 2b of the Bush–Jacoby–Medeiros scheme. Accordingly, its molecular mass (31 kDa) and isoelectric point (9.35) fall within

the range of values (19–36 kDa and 4.9–9.5, respectively) reported for group 2b enzymes (Bush *et al.*, 1995).

### pH-Dependence of $k_{\text{cat}}$ and $K_m$

The effect of pH on  $k_{\text{cat}}$  and  $K_m$  for benzylpenicillin hydrolysis was studied in the pH range 5.5–8.5. Steady-state kinetic parameters could not be evaluated out of this range because (i) the enzyme irreversibly inactivated when pre-incubated at  $\text{pH} \leq 5$  and (ii) a fast and irreversible inhibition was observed when the enzyme was pre-incubated with benzylpenicillin at pH 9.

$k_{\text{cat}}$  decreased with increasing pH values (Fig. 3a) according to Equation 1:

$$k_{\text{cat}} = \frac{k_{\text{cat}}^{\text{ESH}_n} + k_{\text{cat}}^{\text{ESH}_{n+1}} 10^{[\text{p}K_{1(\text{ES})} - \text{pH}]}}{1 + 10^{[\text{p}K_{1(\text{ES})} - \text{pH}]} + 10^{[\text{pH} - \text{p}K_{2(\text{ES})}]}} \quad (1)$$

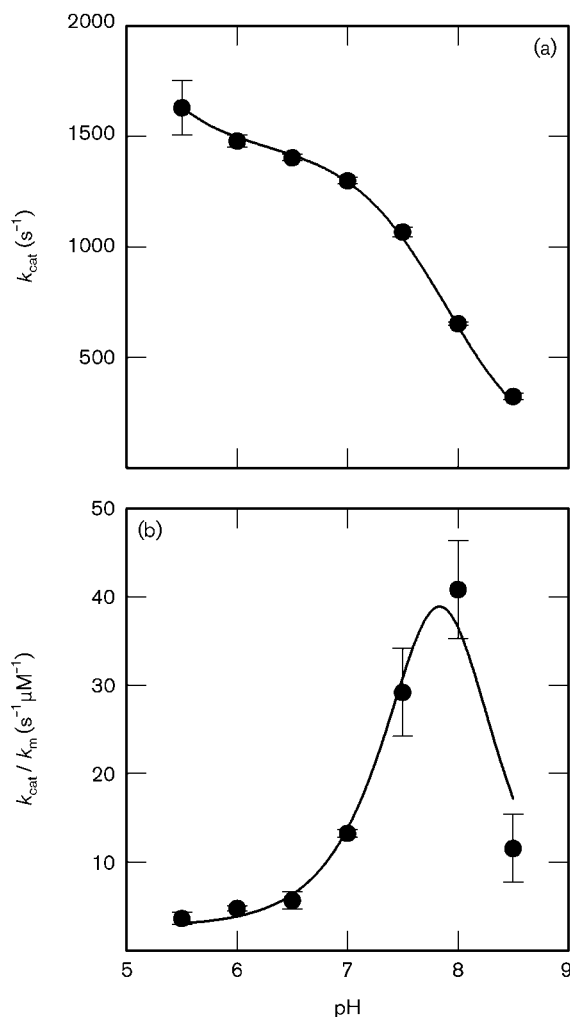
Equation 1 describes the kinetic behaviour of a system that includes three ionic forms of the enzyme–substrate (ES) complex: two ( $\text{ESH}_{n+1}$  and  $\text{ESH}_n$ ) that can yield product and an inactive one ( $\text{ESH}_{n-1}$ ), which is the result of the dissociation [ $\text{p}K_{2(\text{ES})} = 7.9$ ] of a hydrogen ion ( $\text{H}^+$ ) from  $\text{ESH}_n$  [see Scheme I (Fig. 4) and Fig. 3a]. Since no decay of activity was observed in the acid region, it can be presumed that the  $\text{p}K$  value for the general base that has been suggested to play a role in the reaction mechanisms of class A and C  $\beta$ -lactamases (Lamotte-Brasseur *et al.*, 1999; Page & Laws, 1998) is lower than 4.7. A similar behaviour has been reported for the  $k_{\text{cat}}$  dependence on pH of the  $\beta$ -lactamase of *Streptomyces albus* G (Brannigan *et al.*, 1991).

$K_m$  value decreased from 450  $\mu\text{M}$  at pH 5.5 to 30  $\mu\text{M}$  at pH 8.5.  $k_{\text{cat}}/K_m$  exhibited a maximum value at pH 8.0. At lower pH values,  $k_{\text{cat}}/K_m$  decreased to a limiting value significantly different from zero (Fig. 3b) according to Equation 2:

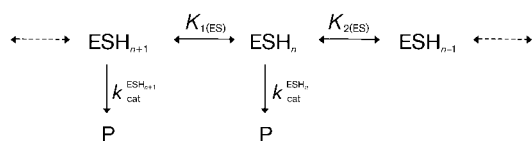
$$\frac{k_{\text{cat}}}{K_m} = \frac{\left(\frac{k_{\text{cat}}}{K_m}\right)^{\text{EH}_{n+1}} + \left(\frac{k_{\text{cat}}}{K_m}\right)^{\text{EH}_n} \times 10^{[\text{p}K_{1(\text{E})} - \text{pH}]}}{1 + 10^{[\text{p}K_{1(\text{E})} - \text{pH}]} + 10^{[\text{pH} - \text{p}K_{2(\text{E})}]}} \quad (2)$$

Equation 2 describes the kinetic behaviour of the system shown in Scheme II (Fig. 5). According to Scheme II, two ionic forms of the free enzyme ( $\text{EH}_{n+1}$  and  $\text{EH}_n$ ) could participate in productive binding of substrate, whereas a third one ( $\text{EH}_{n-1}$ ) would be unable to productively bind substrate. The estimated catalytic efficiency of  $\text{EH}_n$  is two orders of magnitude higher than that of  $\text{EH}_{n+1}$  (Scheme II and Fig. 3b). A similar pH-rate profile has been described for the RTEM-2  $\beta$ -lactamase (Knap & Pratt, 1991). However,  $\beta$ -lactamase from *A. lipoferum* differs from the RTEM-2  $\beta$ -lactamase in the fact that  $\text{p}K_{1(\text{E})} > \text{p}K_{2(\text{E})}$ , which indicates that proton binding is positively cooperative.

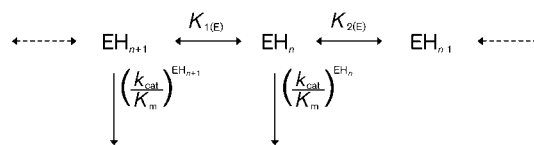
The catalytic efficiency estimated for the  $\text{EH}_n$  form ( $3.7 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ ) of the *A. lipoferum*  $\beta$ -lactamase described here is near the diffusion control limit as it would be expected for a ‘perfect catalyst’. However, the



**Fig. 3.** Effect of pH on kinetic parameters of the  $\beta$ -lactamase from *A. lipoferum*.  $K_m$  and  $k_{cat}$  values were estimated as indicated in Methods at different pH values in a medium containing 30 mM sodium acetate, 30 mM sodium phosphate and 30 mM sodium pyrophosphate, using benzylpenicillin as the substrate. (a)  $k_{cat}$  Values estimated at different pH values; the curve line has been drawn using Equation 1 (see Results) with the following parameters:  $k_{cat}^{ESH_n} = 1450 \text{ s}^{-1}$ ;  $k_{cat}^{ESH_{n+1}} = 2800 \text{ s}^{-1}$ ;  $pK_{1(ES)} = 4.7$ ;  $pK_{2(ES)} = 7.9$ . (b)  $k_{cat}/K_m$  Values estimated at different pH values; the line is the theoretical behaviour described by Equation 2 with the following parameters:  $\left(\frac{k_{cat}}{K_m}\right)^{EH_n} = 3.7 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ ;  $\left(\frac{k_{cat}}{K_m}\right)^{EH_{n+1}} = 2.7 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ ;  $pK_{1(E)} = 8.5$ ;  $pK_{2(E)} = 7.2$ .



**Fig. 4.** Scheme I.



**Fig. 5.** Scheme II.

catalytic efficiency for benzylpenicillin hydrolysis reached an experimental maximum ( $4 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ ) around pH 8.0, which is one order of magnitude lower than that estimated for  $EH_n$ . Therefore, at the optimum pH only 10 % of the enzyme molecules would be in the ionic form with higher catalytic efficiency.

In summary, the high natural resistance of *A. lipoferum* RG20 to  $\beta$ -lactam antibiotics is due to the production of an inducible  $\beta$ -lactamase with an interesting pH-rate dependence and substrate and inhibitor profiles that agree well with those of group 2b  $\beta$ -lactamases of the Bush–Jacoby–Medeiros scheme.

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