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Purification and properties of the *Mycobacterium smegmatis* mc^{2}155 β-lactamase

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Abstract

The β-lactamase of *Mycobacterium smegmatis* mc^{2}155 has been purified to protein homogeneity. Its N-terminal sequence and catalytic properties are similar to those of the β-lactamase produced by *Mycobacterium fortuitum* D316 and establish this new enzyme as a member of molecular class A.

Keywords: *Mycobacterium smegmatis*; *Mycobacterium smegmatis* mc^{2}155 β-lactamase; Purification

1. Introduction

The low permeability of the cell envelope is usually considered to be a major factor in the high resistance of most mycobacteria to antimicrobial agents [1], but the production of antibiotic modifying enzymes also contributes to this phenomenon in both clinical and natural isolates [2].

Accordingly, the low sensitivity of mycobacteria to β-lactam antibiotics [3] rests in part on the cell wall diffusion barrier and the production of β-lactamase. The presence of the latter enzyme has been detected in a large number of species including such redoubtable pathogens as *Mycobacterium tuberculosis* and *Mycobacterium leprae* [4,5]. The contribution of the third factor, the sensitivity of the penicillin binding proteins, has been poorly studied. In this study, the β-lactamase produced by *Mycobacterium smegmatis* mc^{2}155 has been purified, characterized and compared to that produced by *Mycobacterium fortuitum*, the only well studied mycobacterial β-lactamase [6–8].

2. Materials and methods

2.1. Antibiotics

Nitrocefin was purchased from Oxoid (Basingstoke, UK). All other antibiotics were generous gifts of various companies, as described before [9].

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2.2. Bacterial strains and culture conditions

Mycobacterium smegmatis mc^{2}155 [10] was grown on Middlebrook 7H10 agar plates (Difco). For submerged cultures, the following media were used at 37°C: Middlebrook 7H9 broth (Difco); minimal medium: 5 g casamino acids, 10 g glucose, 2 g $(NH_4)_2SO_4$, 3.5 g K_2HPO_4, 0.6 g $MgSO_4$, 50 g PEG 600, 1 ml trace elements (0.1 g/ml $FeSO_4 \cdot 7H_2O$, 0.1 g/ml $MnCl_2$, 0.1 g/ml $ZnSO_4 \cdot 7H_2O$) for 1 liter and Müller-Hinton broth (Oxoid) supplemented with 0.05% Tween 20 and 0.2% glycerol.

For production, 3 ml of a stationary phase culture of *M. smegmatis* mc^{2}155 was used to inoculate 1 liter of medium, which was incubated at 37°C under agitation (250 rpm). The supernatant was collected by centrifugation at $11\,000 \times g$ (30 min at 4°C).

2.3. Assay of protein concentration and enzyme activity

The total protein concentration was measured by the method of Bradford [11]. The enzyme activity was routinely determined by monitoring the hydrolysis of nitrocefin at 482 nm on a microcomputer-linked Hewlett Packard HP 500 spectrophotometer. One unit of activity is defined as the amount of enzyme required to hydrolyse 1 μmol of nitrocefin per minute at 37°C in 50 mM sodium phosphate buffer, pH 7.2 containing 0.2 M KCl and 0.05 mg/ml of bovine serum albumin. The same incubation conditions were used for all kinetic parameter determinations.

2.4. Purification of the β-lactamase

The β-lactamase was produced in Müller-Hinton broth. After centrifugation, the supernatant was collected and concentrated on an Amicon 8400. The concentrated solution (130 ml) was dialyzed against 10 liters of 10 mM Tris-HCl buffer, pH 8. The crude extract was adsorbed on a High Load Q Sepharose High Performance column (36/10 Pharmacia, Uppsala, Sweden) preequilibrated with the same buffer. The enzyme was eluted by a linear NaCl gradient (0–0.5% over 300 ml). The active fractions were pooled and dialyzed against 25 mM Tris-HCl buffer pH 6.3.

The solution was concentrated to 10 ml and further purified by chromatofocusing over the pH range 7–4 on a monoP HR 5/20 column (Pharmacia). The active fractions were concentrated to 2 ml and filtered through a Superdex 75 10/30 column (Pharmacia) to eliminate the polybuffer.

The M_r of the enzyme was determined by SDS/PAGE, the M_r standards were from BioRad Laboratories (Richmond, CA, USA). The isoelectric pH of the β-lactamase was estimated on Ampholine PAGE plates [6] (Pharmacia).

Table 1
Kinetic parameters of the *Mycobacterium smegmatis* β-lactamase

Antibiotic	[Protein] (ng/ml)	[Antibiotic] (μM)	λ (nm)	Δε (M^{-1} s^{-1})	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1} s^{-1}$)
Benzylpenicillin	540	20–1000	232	−800	70 ± 7	46 ± 5	0.66
Oxacillin	540	2–20	260	370	6.5	$k_{+3} = 0.0045 \pm 5 \times 10^{-4}$	$k_{+2}/K = 0.0007 \pm 7 \times 10^{-5}$
Carbenicillin	270	500–2000	235	−830	150 ± 15	12 ± 1	0.08
Imipenem	1080	100–2000	299	−8000	540 ± 50	0.16 ± 0.01	0.03
Nitrocefin	10–540	10–300	482	15000	320 ± 32	1000 ± 90	3
Cephalorodine	270	200–2000	255	−9000	100 ± 10	57 ± 6	0.6
Cephalosporine C	20–540	10–300	262	−6600	130 ± 13	22 ± 2	0.2
Cefuroxime	1350	10–1000	273	−7500	120 ± 12	3 ± 0.3	0.025
Cefotaxime	1350	10–1000	264	−6700	210 ± 20	1 ± 0.1	0.005
Cephalotin	1080	10–1000	260	−6300	70 ± 7	43 ± 4	0.6
Ceftazidime	1350	2000	260	−9000	> 1 mM	ND	ND
Clavulanic acid	1350	1–20			$K_i = 1.7 \pm 0.2$	slow inactivation	ND

ND: not determined.

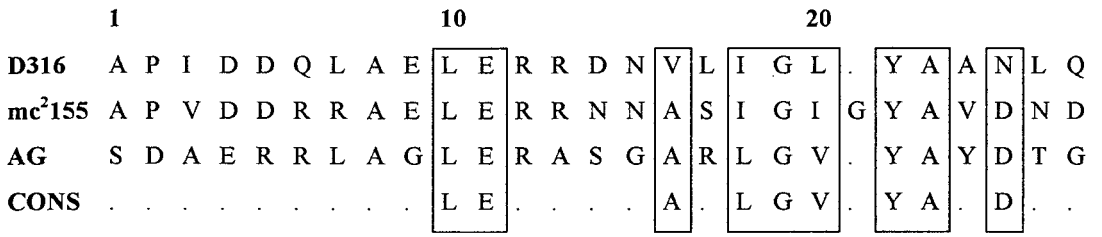

Fig. 1. Comparison of the *Mycobacterium smegmatis* mc²155 (mc²155) β-lactamase N-terminal sequence with those of the *Mycobacterium fortuitum* D316 (D316) and *Streptomyces albus* G (AG) β-lactamases. Residues found in many, but not all, class A β-lactamases are also shown (CONS) [15].

2.5. N-terminal amino acid sequence

The N-terminal amino acid sequence was determined with the help of a 477A pulse-chase sequenator (Applied Biosystems, Foster City, CA, USA). The amino acid phenylthiohydantoin derivatives were identified by reverse phase HPLC on a 120A Analyzer (Applied Biosystems). Twenty-seven automated Edman degradation cycles were performed. The protein (10 nmol) was prepared by SDS/PAGE followed by electrotransfer on an Immobilon-P membrane 5 (Millipore, Bedford, MA, USA) and Coomassie blue staining [12].

2.6. Determination of kinetic parameters

The experimental conditions are described in Table 1. The values represent averages of six different experiments performed with various enzyme and substrate concentrations. The data were collected on a microcomputer-linked Uvikon 80 spectrophotometer [13].

For compounds behaving as substrates, the steady-state parameters k_{cat} and K_m were measured by adding the enzyme to 500 μl of substrate solutions and monitoring complete hydrolysis time courses. Analysis was performed by the method of De Meester et al. [14]. For nitrocefin and cephalosporin C, initial rates were also determined and the kinetic parameters obtained with the help of the Hanes-Woolf linearization of the Henri-Michaelis equation. When the β-lactam was rather stable to β-lactamase action, the k_{cat} value was obtained from the initial rate measured at saturating substrate concentrations and the K_m determined as a K_i in competition experiments with nitrocefin as reporter substrate.

Oxacillin behaved as a transient inactivator. A rather stable acylenzyme (EC*) accumulated and the interaction was studied on the basis of the three-step model.

$$E + C \leftrightarrow^{K} EC \rightarrow^{k_{+2}} EC^{*} \rightarrow^{k_{+3}} E + P$$

where E, C, EC and P are the enzyme, the substrate, the Henri-Michaelis complex and the hydrolysis product, respectively, k_{+2} and k_{+3} the first order acylation and deacylation rate constants, respectively, and K the dissociation constant of EC.

The k_{+3} value was obtained by directly measuring the rate of 1 mM oxacillin hydrolysis at high (1 mg/ml) enzyme concentration. The k_{+2}/K value was derived from the rate of enzyme inactivation with nitrocefin as reporter substrate [14]. From these data, the K_m value was calculated as $k_{+2}/(k_{+3} \times K)$.

2.7. Thermal stability and effects of chaotropic agents

The β-lactamase was incubated at increasing temperatures or in the presence of 1–8 M urea or 1–6 M guanidinium chloride. Aliquots were withdrawn after various periods of time and the residual activity determined. The activity of the enzyme was also measured in the presence of lower concentrations of urea and guanidinium chloride.

2.8. Influence of the pH on the enzyme activity

The k_{cat}/K_m value for cephalothin and the activity versus 100 μM nitrocefin were determined in the pH range 4–11 in the following buffers: pH 4–6: 100

mM cacodylic acid/sodium cacodylate; pH 7: 100 mM sodium phosphate; pH 8–9: 100 mM Tris-HCl; pH 10–11: 100 mM $NaHCO_3/Na_2CO_3$.

3. Results and discussion

3.1. Production, purification, M_r and pI determinations

The best production was obtained in Müller-Hinton broth after 70 h of submerged culture. A three-step procedure yielded a preparation of high purity. Starting with 900 ml of supernatant containing 4.5 mg of protein, 0.2 mg of pure enzyme was recovered with a yield of 20%. An equivalent amount of activity was found to remain cell-bound and could be solubilized by sonication. Although mycobacterial β-lactamases are usually considered cell-bound, *Mycobacterium smegmatis* thus appears to secrete 50% of the enzyme into the supernatant, at least under our culture conditions.

The electrophoretic analysis indicated a homogeneous protein exhibiting a M_r value of $31\,000 \pm 1000$ and an isoelectric pH of 4.5 which is significantly different from that of the *Mycobacterium fortuitum* enzyme [6].

3.2. N-terminal amino acid sequence

Fig. 1 compares the first 24 N-terminal residues of the β-lactamase with the corresponding sequences of the *Mycobacterium fortuitum* and *Streptomyces albus G* class A β-lactamases and demonstrates a high degree of similarity with the former enzyme.

3.3. Kinetic parameters

The enzyme exhibited a rather broad specificity spectrum (Table 1) catalysing the hydrolysis of both cephalosporins and penicillins. Nitrocefin was the best tested substrate.

With oxacillin ($K > 20$ μM; $k_{+2} > 0.014$ s^{-1}) the observed accumulation of the acylenzyme was explained by a high k_{+2}/k_{+3} ratio larger than 1.

Clavulanic acid behaved as a slow inactivator; 50% of the activity was lost after 5 min of incubation at a high (250 μM) concentration of clavulanic acid, and cefotaxime was very poorly recognized.

If one excepts cefuroxime and cefotaxime, on which the *Mycobacterium smegmatis* enzyme was significantly (8–20-fold) less active than its *Mycobacterium fortuitum* counterpart, the kinetic properties of the two enzymes were rather similar including a low sensitivity to inactivation by clavulanic acid [6]. In addition, the *Mycobacterium smegmatis* enzyme cross-reacted with antibodies raised against the *Mycobacterium fortuitum* purified β-lactamase.

3.4. Thermal stability

The half-lives of the β-lactamase at 56°C, 62°C and 70°C were 6, 2.5 and 1 min. respectively. At temperatures higher than 50°C partial reversibility was observed after cooling of the sample. No loss of activity was detected after 12 h of incubation at temperatures below 50°C (data not shown).

3.5. Interaction of the enzyme with chaotropic agents

At concentration above 1 M, guanidinium chloride induced an irreversible denaturation, while denaturation by urea was reversible. After preincubation in 1–6 M urea solutions followed by dilution in the assay mixture the activity was fully recovered. Inhibition was observed when 1 M urea was present in the final assay mixture.

3.6. Influence of the pH on enzyme activity

The activity of the β-lactamase on 100 μM nitrocefin and the k_{cat}/K_m value of cephalothin both exhibited broad maxima between pH 6 and 8. At pH 7 activity was not modified when the concentration of sodium phosphate was increased from 10 to 100 mM.

4. Conclusion

Like its *Mycobacterium fortuitum* counterpart, the *Mycobacterium smegmatis* β-lactamase is a class A β-lactamase [15]. Although significantly less active than the most efficient class A enzymes (TEM, *Bacillus*

licheniformis), it exhibits a rather broad specificity profile and includes several cephalosporins among its best substrates. Both mycobacterial β-lactamases are very slowly inactivated by clavulanic acid. It would be interesting to determine if this property is shared by other mycobacterial β-lactamases and if the catalytic parameters of the enzymes discussed here are representative of those of other mycobacterial β-lactamases.

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