SHORT REPORT

The effect of zinc ions on the activity of metallo- β -lactamases of *Bacteroides fragilis*

R. EDWARDS, P. S. HASHMI and D. GREENWOOD

Division of Microbiology and PHLS Laboratory, University Hospital, Queen's Medical Centre, Nottingham NG7 2UH

The zinc supplement required to achieve the maximum activity of metallo- β -lactamases from 12 *Bacteroides fragilis* isolates was investigated. Changes in absorbance of imipenem in a spectrophotometric assay with crude cell extracts were performed in the presence and absence of various concentrations of zinc sulphate. The greatest degree of imipenem hydrolysis was seen with the addition of between 50 and 500 μ M zinc sulphate, and the degree of stimulation of enzyme activity in the strains tested varied six-fold. Increasing the zinc sulphate concentration resulted in inhibition of hydrolysis with extracts of low enzymic activity. These findings indicate the importance of determining the optimal zinc concentration for each strain tested in kinetic studies of metallo- β -lactamases.

Introduction

Carbapenems, such as imipenem, are commonly highly active against *Bacteroides fragilis* because of the inability of the typical serine- β -lactamases to hydrolyse these antibiotics [1]. However, a minority (2–5%) of *B. fragilis* isolates produce zinc-dependent metallo- β -lactamases that are able to hydrolyse carbapenems [2, 3]. These enzymes are responsible for a wide range of resistance to carbapenems depending on their level of production [4].

Detection, kinetic analysis and inhibition profiles of these enzymes traditionally rely on spectrophotometric examination of the hydrolysis of imipenem with β -lactamase extracts in phosphate buffer. It has been shown that the trace amounts of zinc ions present in these assays is insufficient to achieve maximum hydrolysis of imipenem [5]. In contrast, Munn *et al.* [6] described an inhibitory effect of zinc sulphate > 100 μ M on imipenemase activity of a *B. fragilis* isolate.

This study examined the effect of various concentrations of zinc ions on the hydrolysis of imipenem by metallo- β -lactamases of *B. fragilis* isolates.

Materials and methods

Bacterial strains

Twelve *B. fragilis* isolates known to produce metallo- β -lactamases were examined. Eight of these strains, which showed reduced susceptibility or moderate resistance to imipenem (MIC of imipenem 1–32 mg/L), were clinical isolates from Nottingham Public Health Laboratory. The remaining four strains were highly resistant isolates (MIC 128–512 mg/L) obtained from other centres. *B. fragilis* NCTC 9344 was included as an imipenem-sensitive control.

Preparation of β -lactamase extracts

Bacterial growth from blood agar plates was harvested in 10 ml of phosphate buffer (0.02 M in de-ionised water, pH 7.0) after incubation for 48 h in anaerobic conditions at 37°C. The cells were disrupted by 10 $\times$ 30-s bursts of sonication (Soniprep 150, MSE, Crawley, Sussex) and centrifuged at 4000 rpm for 20 min. The supernates (crude β -lactamase extracts) were stored at –70°C.

Analysis of imipenem hydrolysis

The change in absorbance of imipenem (0.2 ml of a 1 mM solution) at 299 nm was measured with a UV-160A spectrophotometer (Shimadzu, Kyoto, Japan) in the presence of 0.2 ml of crude cell extracts and 0.6 ml of phosphate buffer before and after incubation at 37°C for 1 h. The cell extracts were concentrated by freeze

drying or, in the case of those of the highly resistant strains, diluted with phosphate buffer, to achieve similar hydrolysis rates of imipenem with complete hydrolysis after incubation for 1 h. Experiments were repeated in the presence of zinc sulphate (final concentration) 10, 50, 250, 500, 2500, 5000 and 10 000 μM , in the presence and absence of crude cell extracts. The zinc ion concentration of the assay mixture without zinc sulphate supplement was measured with a flame atomic absorbance spectrophotometer (939 AA Spectrometer, Unicam, Cambridge).

Results and discussion

The results are summarised in Table 1. The residual zinc content of the unsupplemented assay system was low (3.5–6.4 μM). As zinc ions can leach from glass vessels, plastic containers were used where possible [7]. However, glass freeze-drying vials were used with extracts of low or moderate activity and this may account for the marginally higher zinc content in these assays.

The concentration of zinc sulphate required to produce the maximum degree of hydrolysis for all test strains ranged between 50 and 500 μM ; for eight of the 12 strains tested this concentration was 250 μM . The maximum stimulation of imipenem hydrolysis resulting from the addition of zinc ions with strains showing low to moderate resistance to imipenem (MIC 1–32 mg/L) was 27–51% of the hydrolysis observed in the presence of residual zinc but without zinc supplement. For the highly resistant strains (MIC >64 mg/L) these values were greater (62–169%). In the absence of metallo- β -lactamase extracts or in the presence of crude cell extract of *B. fragilis* NCTC 9344, the addition of zinc sulphate failed to produce a change in absorbance with imipenem.

Table 2. The effect of various concentrations of zinc sulphate on the hydrolysis of imipenem by metallo- β -lactamases of *B. fragilis*

Strain no.	Percentage of maximum hydrolysis achieved by ZnSO_4 supplement (μM) of						
	10	50	250	500	2500	5000	10 000
1	10	48	100	62	72	24	-48
2	10	42	100	68	35	10	-77
3	23	97	85	100	80	23	-28
4	35	54	100	70	19	-3	-49
5	43	77	100	97	93	53	-33
6	45	55	100	100	40	-35	-1
7	57	100	100	63	57	51	-33
8	85	96	100	96	93	-41	-37
9	70	96	100	99	90	54	56
10	90	100	94	80	68	49	60
11	76	79	97	100	74	74	72
12	99	100	99	91	88	59	70

For each of the extracts tested, a reduction in hydrolysis was observed as the concentration of zinc sulphate was increased beyond that which allowed maximum activity (Table 2). In the case of isolates for which the MIC of imipenem was ≤ 32 mg/L, high concentrations (5000 or 10 000 μM) inhibited enzyme activity. For the four highly resistant strains, these high zinc sulphate concentrations reduced the maximum stimulation of imipenem hydrolysis by up to 51%.

These results show that metallo- β -lactamases from *B. fragilis* strains respond differently to the presence of various concentrations of zinc ions. A range of zinc concentrations was required to produce maximum stimulation of these metallo-enzymes, the level of stimulation varied between strains and the degree of inhibition resulting from the presence of high concentrations of zinc differed. These inter-strain differences occurred despite attempts to normalise the enzyme activities by adjusting the concentration of

Table 1. The zinc ion concentration associated with the maximum hydrolysis of imipenem by metallo- β -lactamases of *B. fragilis*

Strain no.	MIC of imipenem (mg/L)	Zn^{2+} (μM) in assay*	ZnSO_4 supplement (μM) causing maximum hydrolysis	Increase in hydrolysis (%)†
1	1	5.3	250	29
2	1	5.3	250	31
3	4	5.2	500	33
4	2	6.4	250	37
5	1	4.0	250	30
6	4	3.8	250/500	37
7	4	4.8	50/250	51
8	32	4.6	250	27
9	128	3.7	250	169
10	256	3.5	50	109
11	256	3.7	500	62
12	512	3.5	50	101

*Residual concentration without ZnSO_4 supplement.

†Percentage increase in hydrolysis achieved by the concentration of zinc sulphate causing maximum hydrolysis.

the crude β -lactamase extracts. As standardised methods were used, the range of activity of metallo- β -lactamases from different strains observed in the presence of zinc suggests biological diversity among this group of enzymes. The stimulation and inhibition of metallo- β -lactamase activity with zinc sulphate reported by Munn *et al.* [6] occurred with markedly lower concentrations than observed in this study, and this may be accounted for by differences in the assay conditions as well as strain variation.

This study showed that hydrolysis of imipenem by metallo- β -lactamases present in crude cell extracts of *B. fragilis* can be enhanced by the addition of zinc sulphate, and by further increasing the zinc sulphate concentration inhibition of these enzymes occurs. The concentration of zinc sulphate required to achieve maximum stimulation, together with the degree of enhancement, varies from strain to strain. These findings indicate the importance of determining the optimal zinc ion concentration for each strain when kinetic studies are undertaken.

We thank Mr J. Hughes of the Clinical Chemistry Department, Queen's Medical Centre, Nottingham for the analysis of zinc concentrations by flame atomic absorbance spectrophotometry.

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