Some Properties of Two Cell-Bound β -Lactamases from *Bacillus cereus* 569/H

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The purification and properties of two extracellular β -lactamases from *Bacillus cereus* 569/H, named β -lactamase I and β -lactamase II, were described briefly by Kuwabara & Abraham (1967). β -Lactamase I appears to be identical with the extracellular α -penicillinase isolated by Kogut, Pollock & Tridgell (1956), whereas β -lactamase II is a Zn^{2+} -requiring enzyme active against a variety of cephalosporins as well as penicillins.

Two cell-bound β -lactamases were reported by Pollock (1956) to be produced by *B. cereus* 569/H and were named β -penicillinase and γ -penicillinase respectively. β -Penicillinase was thought to be identical with α -penicillinase and to be bound near the surface of the cells. γ -Penicillinase was distinguishable immunologically from α -penicillinase and showed a higher solubility than the latter in saturated $(\text{NH}_4)_2\text{SO}_4$ solution. With the discovery of β -lactamase II it became of interest to ascertain whether any Zn^{2+} -requiring enzyme was cell-bound.

Determinations of units of β -lactamase activity, protein and protein N were made as described by Kuwabara & Abraham (1967).

B. cereus 569/H was grown as described by Miller, Bach & Markus (1965), except that the casein-hydrolysate medium used for growth contained ZnSO_4 (1 mM). The cells were separated from culture fluid by centrifugation and washed at the centrifuge (3000g for 45 min.) with a volume equal to the corresponding volume of culture fluid of 1 M-NaCl-0.1 M-sodium citrate solution that had been adjusted to pH 8.5. They were then repeatedly washed with approx. 3 ml. of this solution/ml. of packed cells until the washing contained less than 50 units of β -lactamase I activity/ml. The washed cells were stored at -15° .

An acetone-dried powder was prepared from the cells by stirring them with 23 times (v/w) their amount of acetone at -15° . The weight of the powder was 25% of that of the wet cells. Cell-free extracts were obtained by grinding the powder at 4° with sand and a 20-fold (v/w) amount of water or low-ionic-strength tris-acetate buffer, pH 7.0 (0.4 μM with respect to tris), which in each case contained 1 mM- ZnSO_4 when the extract was to be used for the study of cephalosporinase activity. The extracts contained 6140 units of penicillinase/mg. of protein N, which was 3.6% of that in the amount of the extracellular fluid from which the

cells had been derived. Their cephalosporinase activity was 570 units/mg. of protein N, and was 13.3% of that in the corresponding quantity of extracellular fluid. Preliminary experiments indicated that the cephalosporinase activity was entirely Zn^{2+} -dependent, but that the penicillinase activity was not.

Nucleic acid was removed from the cell-free extract (200 ml.) by precipitation with streptomycin sulphate (final concn. 1.75%, w/v) and treatment of the supernatant with crystalline deoxyribonuclease and ribonuclease (each 2 mg.) for 30 min. at 35° . Material obtained by fractional precipitation with $(\text{NH}_4)_2\text{SO}_4$ (61–89% saturation) was dialysed at 4° against 40 mM-tris-acetate buffer and fractionally precipitated by addition of acetone at -10° . The precipitate obtained when the amount of acetone added was raised from 30% to 100% (v/v) was dialysed against 0.4 mM-tris-acetate buffer, pH 7.0, and chromatographed on a column of DEAE-cellulose (60 cm. $\times$ 1.5 cm. diam.). Elution was with an NaCl gradient obtained by addition of M-NaCl-0.4 mM-tris-acetate buffer, pH 7.0, to a reservoir containing 500 ml. of stirred 0.4 mM-tris-acetate buffer, pH 7.0, and 3.8 ml. fractions were collected. The β -lactamase eluted in fractions 18–23 showed penicillinase activity (316 000 units/mg. of protein N with benzylpenicillin as substrate, overall yield 52%) but negligible cephalosporinase activity (< 20 units/mg. of protein N).

A Zn^{2+} -dependent β -lactamase with both cephalosporinase and penicillinase activity was obtained by a similar procedure except that all the buffers used in the purification contained 1 mM- ZnSO_4 and that fractional precipitation with $(\text{NH}_4)_2\text{SO}_4$ was followed by selective denaturation of the enzyme with only penicillinase activity at 65° (Kuwabara & Abraham, 1967). The enzyme was eluted from the DEAE-cellulose column in fractions 7–11. The specific activity of the enzyme (cephalosporin C as substrate) was 116 000 units/mg. of protein N (overall yield 48%).

Table 1 shows the behaviour of the two preparations of cell-bound enzymes to a variety of penicillin and cephalosporin substrates in comparison with that of extracellular β -lactamase I and β -lactamase II. The cell-bound enzyme with only penicillinase activity resembled closely extracellular β -lactamase I in its enzymic properties and is subsequently

Table 1. *Maximum rates of hydrolysis and Michaelis constants for different penicillins and cephalosporins with both cell-bound and extracellular β -lactamase I and β -lactamase II*Values of $V_{\max}$ and K_m were obtained from Lineweaver-Burk plots.

Enzyme	Substrate	Extracellular		Cell-bound	
		$V_{\max}$ (relative)	K_m (mM)	$V_{\max}$ (relative)	K_m (mM)
β -Lactamase I	Benzylpenicillin	100	0.037	100	0.038
	Phenoxyethylpenicillin	102	0.061	98	0.063
	Ampicillin	100	1.3	101	0.3
	Penicillin N	38	0.053	31	0.06
	Methicillin	< 1.0	—	< 1.0	—
	Oxacillin	< 2.6	—	< 2.6	—
	Cephalosporin C	< 0.1	—	< 0.1	—
	Cephalothin	< 0.1	—	< 0.1	—
	Cephaloridine	< 0.1	—	< 0.1	—
	Cephaloglycin	< 0.1	—	< 0.1	—
	Deacetylcephalosporin C	< 0.1	—	< 0.1	—
	Deacetylcephalosporin C lactone	30	2.0	30	2.0
	Deacetoxycephaloglycin	< 0.1	—	< 0.1	—
β -Lactamase II	Benzylpenicillin	100	3.3	100	3.3
	Ampicillin	64	3.3	75	3.3
	Methicillin	89	1.6	111	2.2
	Oxacillin	89	1.6	112	3.3
	Cloxacillin	89	2.3	83	1.7
	Quinacillin	10*	—	9*	—
	Cephalosporin C	80	1.1	75	1.4
	Cephalothin	89	1.0	67	3.3
	Cephaloridine	41	3.3	56	2.0
	Cephaloglycin	50	0.5	67	2.5
	Deacetylcephalosporin C	< 0.5†	—	< 0.5†	—
	Deacetylcephalosporin C lactone	< 0.5	—	< 0.5	—
	Deacetoxycephaloglycin	< 0.5	—	< 0.5	—

* Relative rate of hydrolysis with substrate concentrations 3.0 mM.

† Relative rate of hydrolysis with substrate concentration 1.0 mM.

referred to as cell-bound β -lactamase I. For the same reason the enzyme with penicillinase and cephalosporinase activity is referred to as cell-bound β -lactamase II. Among the substrates tested, the three that are resistant to hydrolysis by β -lactamase II are deacetoxy- and deacetyl-cephalosporins, which have no leaving group at C-3.

Cell-bound β -lactamase I is presumably the β -penicillinase described by Pollock (1956). Preliminary attempts to relate the cell-bound β -lactamase II obtained in the present work to γ -penicillinase had no clear outcome. The relative values of $V_{\max}$ with benzylpenicillin and cephalosporin C as substrates and the Michaelis constants, K_m , with benzylpenicillin were similar for cell-bound β -lactamase I to those reported for γ -penicillinase (Pollock, 1956), but were different from those found for β -lactamase II. No cofactor appeared to be needed by cell-bound β -lactamase I and none was reported to be required by γ -penicillinase,

whereas Zn^{2+} was essential for the activity of cell-bound β -lactamase II. However, the cell-bound (and also extracellular) β -lactamase II resembled γ -penicillinase in having a higher solubility in $(NH_4)_2SO_4$ solution than the exo- α -penicillinase described by Kogut *et al.* (1956), and a higher relative activity against methicillin, quinacillin and cephaloridine (Pollock, 1956; Ron-Zenziper & Citri, 1963; Citri & Kalkstein, 1967), although it showed a wider range of activity than that reported for γ -penicillinase against other penicillins and cephalosporins. Iodine (0.33–15 mM) completely inactivated γ -penicillinase (Pollock, 1956), but it had no effect on the activity of cell-bound β -lactamase I, or of β -lactamase II under similar conditions in the presence of Zn^{2+} . However, iodine (1.32 mM) destroyed irreversibly the ability of Zn^{2+} -free cell-bound β -lactamase II to be reactivated on addition of Zn^{2+} .

An assessment of the significance of the apparent

similarities and differences between β -lactamase II and γ -penicillinase is complicated by the fact that the properties of the latter were determined before Zn^{2+} was known to be a cofactor for a β -lactamase from *B. cereus* and before it was added to the culture medium. If γ -penicillinase required Zn^{2+} the reported absence of this enzyme from the extracellular fluid (Pollock, 1956) might have been due to the presence of only trace amounts of Zn^{2+} that were concentrated in the cells. In the present experiments the failure to add Zn^{2+} to the medium resulted in a substantial decrease in the amount of exo- β -lactamase II detected, even when Zn^{2+} was added before assay, and the report by Sabath & Finland (1968) that the enzyme was less stable in the absence of Zn^{2+} was confirmed. If some of the preparations of γ -penicillinase had also contained some β -penicillinase the results of earlier work could perhaps be reconciled with those reported

here. However, further studies are required to clarify the situation.

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