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Biochemical Characterization of the POM-1 Metallo-β-Lactamase from *Pseudomonas otitidis*

Luisa Borgianni,[a] Filomena De Luca,[a] Maria Cristina Thaller,[b] Yunsop Chong,[c] Gian Maria Rossolini,[a,d,e] Jean-Denis Docquier[a]

Dipartimento di Biotecnologie Mediche, Università di Siena, Siena, Italy[a]; Dipartimento di Biologia, Università di Roma Tor Vergata, Rome, Italy[b]; Department of Laboratory Medicine, Yonsei University College of Medicine, Seoul, South Korea[c]; Department of Experimental and Clinical Medicine, University of Florence,[d] and Clinical Microbiology and Virology Unit, Florence Careggi University Hospital,[e] Florence, Italy

The POM-1 metallo-β-lactamase is a subclass B3 resident enzyme produced by *Pseudomonas otitidis*, a pathogen causing otic infections. The enzyme was overproduced in *Escherichia coli* BL21(DE3), purified by chromatography, and subjected to structural and functional analysis. The purified POM-1 is a tetrameric enzyme of broad substrate specificity with higher catalytic activities with penicillins and carbapenems than with cephalosporins.

Metallo-β-lactamases (MBLs) are resistance determinants of clinical importance due to their constant and potent carbapenemase activity, which is usually associated with broad substrate specificity covering most β-lactam families. MBLs may be found either as acquired enzymes encoded by mobile genetic elements or as resident enzymes in some bacterial species. The latter have been detected in some environmental species, of which only some are of clinical significance (1).

POM-1 is a subclass B3 MBL recently identified in *Pseudomonas otitidis* (2), a *Pseudomonas* species that is found associated with otic infections in humans and can also be found as a free-living aquatic *Pseudomonas* species in some environments (3–5). As such, POM-1 represents the first example of a resident MBL found in a pathogenic *Pseudomonas* species (2).

Preliminary functional characterization of POM-1, carried out by expression of the cloned gene in *Escherichia coli* and *Pseudomonas aeruginosa*, revealed an overall broad substrate specificity, with an apparent preference for penicillins and carbapenems (2). However, the biochemical properties of this enzyme, which exhibits the closest similarity (60% of amino acid identity) with the resident L1 MBL from *Stenotrophomonas maltophilia* while being quite divergent from the other subclass B3 enzymes (identity range, 23 to 37% with GOB-1 and BJP-1, respectively), have not been investigated. The aim of this work was to purify and determine the kinetic and other biochemical properties of the POM-1 MBL.

The open reading frame encoding POM-1 was amplified by PCR using the primers POM-1_EXP/f (5′-TTCATATGCGTACCCTGACCCCCC) and POM-1_EXP/r (5′-CGGGATCCTGCGTCATCAGAGACCTC) containing an NdeI and a BamHI restriction site, respectively (underlined). The source of the bla_{POM-1} gene was *P. otitidis* strain MC10330 (2). The NdeI-BamHI fragment was cloned into the pLBII vector (6) and the pET-9a expression vector (Novagen, Inc., Madison, WI) to obtain the recombinant plasmids pLBII-POM-1 and pET-POM-1, respectively. The cloned bla_{POM-1} gene was sequenced to exclude the presence of any PCR-generated mutations.

Escherichia coli XL1-Blue was transformed with the plasmid pLBII-POM-1, and the MIC values of various β-lactam antibiotics were determined using the broth microdilution method as recommended by the CLSI (7) (Table 1). Production of the MBL conferred resistance to ampicillin, cephalothin, and cefoxitin and decreased susceptibility to most other cephalosporins and all tested carbapenems. Piperacillin, ceftazidime, and cefepime MICs were increased by only a single $\log_2$ dilution with the POM-1-producing *E. coli* laboratory strain, suggesting that some of these compounds might behave as poor substrates of the enzyme. The aztreonam MIC value was not affected, as monobactams are not substrates of MBLs.

POM-1 was produced using a T7 promoter-based *E. coli* BL21(DE3), in which the pET-POM-1 plasmid was introduced by electroporation, after growth at 37°C in ZYP-5052 medium (8) supplemented with 50 μg/ml kanamycin for 48 h. The production of β-lactamase activity was monitored by following the hydrolysis of 150 μM imipenem in 50 mM HEPES buffer supplemented with 50 μM $ZnSO_4$ (pH 7.5) (buffer H). POM-1 was purified from the culture supernatant by chromatography. Briefly, the sample was concentrated by ultrafiltration, using a 10,000-nominal-molecular-weight-limit (NMWL) cellulose membrane (Millipore Corporation, Billerica, MA, USA), desalted in a HiPrep desalting column (GE Healthcare) against 10 mM triethanolamine buffer (pH 7.5), and loaded onto a HiTrap Q Sepharose Fast Flow (GE Healthcare) column, previously equilibrated with the same buffer. Proteins were eluted using a solution of 0.08 M NaCl, and fractions containing β-lactamase activity were pooled, concentrated, and stored at −20°C. This protocol yielded approximately 8 mg/liter POM-1, showing a purity of ≥95% (specific activity, 528 ± 55 μmol of imipenem/min · mg of protein). The authenticity of the enzyme preparation was confirmed by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry (calibrated using Protein Standard I [Bruker Daltonics, Bre-

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Address correspondence to Jean-Denis Docquier, jddocquier@unisi.it.

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TABLE 1 *In vitro* susceptibility profile of *E. coli* XL1-Blue carrying the cloned bla_{POM-1} gene (pLBII-POM-1 plasmid vector) in comparison with that of *E. coli* XL1-Blue carrying the empty plasmid (pBC-SK)

β-Lactam	MIC (μg/ml) for:	
	E. coli XL1-Blue (pLBII-POM-1)	*E. coli* XL1-Blue (pBC-SK)
Ampicillin	64	2
Piperacillin	2	1
Ticarcillin	32	8
Cephalothin	64	16
Cefoxitin	64	16
Cefotaxime	0.5	0.12
Ceftazidime	0.5	0.25
Ceftriaxone	0.5	0.06
Cefepime	0.25	0.125
Cefotetan	4	0.5
Imipenem	1	0.125
Panipenem	1	0.25
Meropenem	1	0.06
Ertapenem	1	≤0.015
Doripenem	0.125	≤0.015
Aztreonam	0.25	0.25

men, Germany] supplemented with 0.3 mg/ml hen lysozyme [Sigma-Aldrich] and 0.4 mg/ml purified VIM-2 MBL [9]), which yielded a molecular mass of 28,636.2 Da, in agreement with the expected theoretical value (28,639.5 Da), calculated for the mature protein (signal peptide, residues 1 to 19).

Gel filtration chromatography (9) was used to compute an apparent molecular mass of the enzyme in solution of 115,000 ± 4,000 Da, consistent with a tetrameric quaternary structure, as already described for the *S. maltophilia* L1 MBL (10). Interestingly, the oligomerization of POM-1 was observed despite the presence of a polar Gln residue at position 175, where a Met is found in L1, which was found to interact with a small hydrophobic cavity formed by residues Leu154 (Ile in POM-1), Pro198, and Tyr254 (Phe in POM-1) of the facing subunit. With a spectrophotometric assay with 4-(2-pyridylazo)resorcinol (PAR) as previously described (11), 2.06 ± 0.14 equivalents of zinc were released upon enzyme denaturation, indicating that purified POM-1 is a dizinc enzyme.

The steady-state kinetic parameters of the POM-1 enzyme were determined spectrophotometrically for several substrates at 30°C using buffer H as previously reported (9). The kinetic parameters for a representative group of β-lactam antibiotics are presented in Table 2.

Purified POM-1 showed the highest catalytic efficiencies with ampicillin, cephalothin, and all tested carbapenems (k_{cat}/K_m of $>10^6\ M^{-1}\ s^{-1}$), being better substrates than ticarcillin, piperacillin, or oxyiminocephalosporins. Most other β-lactam compounds behaved as good substrates, with k_{cat}/K_m values of $>10^5\ M^{-1}\ s^{-1}$, the exceptions being ceftazidime and cefepime, which were hydrolyzed 1,680- and 740-fold less efficiently than cephalothin, respectively. The hydrolytic efficiencies of POM-1 with cephalosporins were highly variable, showing values of k_{cat}/K_m ranging from 5.3×10^3 to $8.9 \times 10^6\ M^{-1}\ s^{-1}$. Cephalothin was the best substrate among the tested cephalosporins, due to both a high turnover rate (the highest measured for the tested cephalosporins) and a low K_m value (among the lowest measured for the tested β-lactams).

TABLE 2 Kinetic parameters of the purified POM for the hydrolysis of various β-lactam substrates, in comparison with those of other subclass B3 MBLs[a]

Substrate	k_{cat} (mean ± SE) (s^{-1})			K_m (mean ± SE) (μM)			k_{cat}/K_m ($M^{-1}\ s^{-1}$)									
	POM-1	L1	AIM-1	POM-1	L1	AIM-1	POM-1	L1	AIM-1	SMB-1	CAR-1	THIN-B	CAU-1	BJP-1	FEZ-1	GOB-1
Ampicillin	190 ± 6	175	594	23 ± 3	40	41	8.1×10^6	4.4×10^6	1.4×10^6	2.4×10^6	3.2×10^3	3.7×10^5	5.0×10^5	1.9×10^4	1.1×10^4	—[b]
Ticarcillin	190 ± 6	375	—	500 ± 46	140	—	3.9×10^5	2.7×10^6	—	—	1.3×10^4	—	—	—	1.3×10^4	5.2×10^5
Piperacillin	9 ± 0.3	140	337	53 ± 8	20	17	1.7×10^5	7.0×10^6	2×10^7	1.8×10^5	2.6×10^4	2.0×10^5	5.7×10^5	6.7×10^4	1.2×10^4	1.7×10^6
Cephalothin	60 ± 0.5	—	529	7 ± 0.3	—	38	8.9×10^6	—	1.4×10^7	1.9×10^6	1.2×10^6	—	4.3×10^5	5.8×10^5	2.5×10^6	6.7×10^5
Cefotaxime	56 ± 2	66	609	63 ± 6	26	49	9.0×10^5	2.6×10^6	1.2×10^7	8.9×10^5	1.5×10^6	2.0×10^6	—	1.4×10^5	2.4×10^6	8.5×10^5
Ceftazidime	>4	—	7	>800	—	148	5.3×10^3	1.8×10^5	4.9×10^4	7.7×10^4	2.9×10^5	1.4×10^5	2.0×10^3	4.3×10^3	4.0×10^3	7.6×10^5
Cefepime	>1.7	>15	93	>140	>1,000	594	1.2×10^4	1.9×10^4	1.6×10^5	3.6×10^3	—	7.9×10^3	—	2.0×10^2	6.0×10^3	2.0×10^5
Imipenem	312 ± 7	75	1,700	52 ± 3	100	97	6.0×10^6	7.5×10^5	1.7×10^7	3.9×10^6	—	1.5×10^6	2.0×10^5	6.0×10^4	2.0×10^5	6.6×10^5
Panipenem	500 ± 26	—	—	102 ± 4	—	—	4.9×10^6	—	—	—	—	—	—	—	—	—
Meropenem	220 ± 3	45	1,000	16 ± 1	10	163	1.4×10^7	4.5×10^6	6.8×10^6	4.2×10^6	9.6×10^2	5.0×10^6	2.6×10^5	8.3×10^5	5.0×10^5	5.3×10^6
Ertapenem	69 ± 6	—	—	4 ± 0.5	—	—	1.5×10^7	—	—	—	—	—	—	—	—	—
Doripenem	51 ± 4	—	—	7 ± 0.7	—	—	6.8×10^6	—	—	—	—	—	—	—	—	—

[a] Data for the subclass B3 MBLs are from references 12 and 13 (L1), 14 (THIN-B), 15 (AIM-1), 16 (SMB-1), 17 (GOB-1), 18 (FEZ-1), 19 (BJP-1), 20 (CAU-1), and 21 (CAR-1). Individual kinetic parameters are the means of three measurements.

[b] —, not reported.

POM-1 shows pseudo-first-order kinetics with ceftazidime and cefepime at concentrations up to 140 and 800 μM, respectively, indicating that these substrates were poorly recognized by the enzyme, as a potential consequence of the presence of a positively charged R2 substituent that might negatively impact binding of the substrate in the enzyme active site.

POM-1 shows notably high catalytic efficiencies with carbapenems. Imipenem and meropenem were hydrolyzed more efficiently by POM-1 than by other subclass B3 enzymes (with the exception of AIM-1 for imipenem), with 2- to 14,000-fold higher k_{cat}/K_m values, mostly due to rather high turnover rates (some of which are noticeably higher than those observed with the globally spread acquired MBL VIM-2 [9]) and small K_m values, which also indicated that carbapenems are the preferred substrates of this enzyme.

These kinetic data are overall in agreement with the *in vitro* susceptibility data obtained with the POM-1-producing *E. coli* strain. For instance, the high turnover rates observed for the hydrolysis of ampicillin and ticarcillin translated into more noticeable variations of ampicillin and ticarcillin MIC values between the β-lactamase-producing strains and the parental strain (Table 1). Accordingly, the low k_{cat} value for piperacillin (9 s^{-1}), despite a 10-fold lower K_m value than that of ticarcillin, determines only a modest increase in the MIC values between the two strains (Table 1). As already observed with *E. coli* laboratory strains carrying cloned MBL genes (6), the production of POM-1 resulted in decreased susceptibility, rather than resistance, to carbapenems. This is likely due to the fast permeation of these antibiotics in this organism (22).

In order to better understand the contribution of POM-1 production in relation to the *Pseudomonas otitidis* resistance profile, several attempts to obtain a bla_{POM-1} knockout mutant were carried out but proved unsuccessful. However, enzyme assays did show that >96% of the β-lactamase activity produced by *P. otitidis* strain MC10330, as measured with the chromogenic substrate nitrocefin, which is readily hydrolyzed by most, if not all, β-lactamases, was actually inhibited in the presence of EDTA. In addition, an isoelectric focusing analysis of a crude extract from the same strain did reveal a single nitrocefin-hydrolyzing band at pH 6.0, in agreement with the predicted isoelectric point (pI of 5.98). Overall, these data suggest that POM-1 might represent the major β-lactamase produced by this organism.

In conclusion, POM-1 is a novel broad-spectrum subclass B3 MBL, which exhibits high catalytic activity on carbapenems, including the most recent compounds such as ertapenem and doripenem. It might be regarded as the first member of a group of chromosomal B3 MBLs possibly present within some *Pseudomonas* species, given that a similar enzyme (PAM-1, 72% similar at the sequence level) has been recently reported in *Pseudomonas alcaligenes* (23).

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