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Purification and Biochemical Characterization of IMP-13 Metallo-β-Lactamase▽

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The IMP-13 metallo-β-lactamase was overproduced in *Escherichia coli* BL21(DE3) and purified by chromatography. Analysis of kinetic parameters revealed some notable differences with other IMP-type enzymes, noteworthily a higher catalytic efficiency toward ticarcillin and piperacillin and a marked preference for imipenem over meropenem.

Metallo-β-lactamases (MBLs) are characterized by their ability to efficiently hydrolyze carbapenems and by their insensitivity to the commercially available β-lactamase inhibitors (10). Several types of acquired MBLs have been described worldwide for Gram-negative nonfermenters (GNNFs), among which the IMP- and VIM-type enzymes are the most widespread (9, 10). The IMP-13 MBL has 92.3% amino acid sequence identity with IMP-2 and 82.5% with IMP-1, being quite divergent from other variants. It has been identified in *Pseudomonas aeruginosa* clinical isolates from different countries in Europe and South America, sometimes associated with nosocomial outbreaks (2, 5, 8, 12, 13, 15, 16). The aim of this study was to purify and analyze the kinetic parameters of IMP-13 MBL.

The $bla_{\text{IMP-13}}$ open reading frame was amplified by PCR using primers IMP13-F (5′-CCGAATTCATATGAAGAAAT TATTTGTTTTATGT-3′, containing the EcoRI and NdeI restriction sites) and IMP13-R (5′-AGGGATCCTTAGTTACT TGGTGATGATGTTTT-3′, containing the BamHI restriction site). The source of the $bla_{\text{IMP-13}}$ gene was a *P. aeruginosa* isolate from Argentina (13). The NdeI-BamHI fragment was cloned into the pLBII vector (1) and into the pET-9a expression vector (Novagen, Inc., Madison, WI) to obtain recombinant plasmids pLBII-IMP13 and pET-IMP13, respectively. The authenticity of the cloned fragments was confirmed by sequencing. *Escherichia coli* DH5α (Gibco Life Technologies, Gaithersburg, MD) was transformed with the pLBII-IMP13 plasmid, and the MICs of a representative panel of β-lactam antibiotics were determined at 37°C in Mueller-Hinton broth using the broth microdilution test according to the CLSI (3). The pET-IMP13 plasmid was introduced into *E. coli* BL21(DE3) (Novagen) to overproduce the enzyme. *E. coli* BL21(DE3)(pET-IMP13) was grown overnight in P0.5G noninducing broth, and 1.5 ml of the culture was then inoculated in 1 liter of autoinducing ZYP-5052 medium containing 100 μg/ml of kanamycin (14). The highest specific activity, measured spectrophotometrically with total enzyme extracts, using 200 μM imipenem as the substrate, were achieved in the supernatant fraction of the autoinducing broth (being 2- to 4-fold higher than that obtained in the whole-cell extract at 24 and 48 h, respectively), while the total activities in the supernatant and the cellular fraction were roughly similar.

Enzyme purification was carried out as follows. The supernatant fraction was clarified with solid ammonium sulfate at a final concentration of 40% saturation. The enzyme was then precipitated with addition of solid ammonium sulfate to 75% saturation. After centrifugation, the isolated precipitate was resuspended in 40 ml of 50 mM HEPES buffer containing 50 μM $ZnSO_4$ (pH 7.0) (HB buffer) and the sample was loaded (flow rate, 2 ml/min) onto a 5-ml HiTrap SP HP column (GE Healthcare, Uppsala, Sweden) equilibrated with HB buffer. Elution was performed using a linear NaCl gradient (0 to 1 M). Fractions containing β-lactamase activity were pooled, concentrated 10-fold using a Centriplus ultrafiltration device (YM10 membrane; Millipore, Bedford, MA), and then injected onto an XK 16/70 column packed with 120 ml of Superdex 75 prep grade gel (GE Healthcare) previously equilibrated with HB supplemented with 0.15 M NaCl. Elution was performed with the same buffer at a flow rate of 0.8 ml/min. Fractions containing β-lactamase activity were pooled and stored at −20°C until use. This protocol yielded approximately 1 mg of pure enzyme (final concentration, 2.4 μM), with an overall yield of 53%. The degree of purity was at least 99% as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (6). The authenticity of the enzyme preparation was confirmed by a peptide mass fingerprint matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) analysis (data not shown).

The kinetic parameters of IMP-13 were determined at 37°C in HB (pH 7.5) as previously described (4). Low K_m values were measured as K_i values in competition experiments using 200 μM imipenem as the reporter substrate (7). Purified IMP-1 was used to compare some kinetic parameters in the assayed conditions in this study. The k_{cat} and K_m values for a representative group of β-lactam antibiotics are shown in Table 1. Amino- and ureidopenicillins appeared to be good substrates of IMP-13, while hydrolysis of temocillin, the 6-α-methoxy semisynthetic derivative of ticarcillin, could not be detected. Hydrolysis of piperacillin and, even more significantly, ticarcillin displayed evident differences compared to the

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TABLE 1. Kinetic parameters of purified IMP-13 and comparison with the kinetic parameters of other IMP-type enzymes[a]

Substrate	IMP-13			IMP-1[b]			IMP-2[c]		
	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)
Ampicillin	82 ± 7	210 ± 38	0.38	950	200	4.8	23	110	0.21
Piperacillin[d]	280 ± 40	150 ± 23	1.9	311	356	0.87	–	–	–
Ticarcillin	220 ± 30	130 ± 20	1.8	1.1	740	0.0015	–	–	–
Temocillin		ND	ND	ND	>2,000	<0.0001	–	–	–
Cephalotin	25 ± 1	31 ± 6	0.81	48	21	2.4	–	–	–
Cefoxitin	35 ± 1	12 ± 2	2.9	16	8	2	7	7	1.0
Ceftazidime	9 ± 1	15 ± 3	0.60	8	44	0.18	21	111	0.19
Cefuroxime	23 ± 2	1 ± 0.1[e]	23	8	37	0.22	–	–	–
Cefotaxime	33 ± 2	21 ± 2	1.6	1.3	4	0.35	–	–	–
Cefepime	12 ± 1	8 ± 1	1.5	7	11	0.66	4	7	0.57
Imipenem[d]	120 ± 5	49 ± 5	2.5	42	24	1.8	22	24	0.92
Meropenem[d]	1.4 ± 0.1	10 ± 2	0.14	17	26	0.65	1	0.3	3.3
Ertapenem[d]	1.8 ± 0.1	0.8 ± 0.1[e]	2.2	16	21	0.76	–	–	–

[a] –, data not available; NH, no hydrolysis detected with the use of an enzyme concentration of 220 nM; ND, data could not be determined.
[b] For kinetic data, see reference 7.
[c] For kinetic data, see reference 11.
[d] Kinetic parameter values of IMP-1 for piperacillin, imipenem, meropenem, and ertapenem were determined in this study using the same assay conditions.
[e] K_m was obtained as the K_i value.

other IMP-type variants. All assayed cephalosporins were hydrolyzed by IMP-13. The highest efficiency was observed with cefuroxime, for which the hydrolysis efficiency of IMP-13 was 100-fold higher than that of IMP-1 (Table 1). All tested carbapenem compounds behaved as good substrates, but catalytic efficiency for meropenem was lower than that for imipenem and ertapenem. Unlike with other IMP-type enzymes, the IMP-13 k_{cat}/K_m ratio for imipenem was nearly 20-fold higher than that for meropenem, due to a higher catalytic turnover for imipenem. It is interesting to note that the k_{cat} value for imipenem was also higher than that for IMP-2, which shares the highest amino acid sequence identity with IMP-13 (Table 1). Although further structure-function relationship studies are required, IMP-13 might owe its unique functional features to the presence of 11 unique substitutions. Noteworthily, and on the basis of the IMP-1 structure (Protein Data Bank [PDB] code 1DDK), some substitutions (e.g., Gly177Glu and Val266Lys) might possibly impact on the enzyme properties, as these substitutions might require subtle changes in the protein structure to accommodate the longer side chains of these residues (e.g., the side chain of IMP-13 Lys 266 would clash with the side chains of Leu48 and Val54 in the IMP-1 structure).

TABLE 2. *In vitro* susceptibility of *E. coli* DH5α strains carrying the cloned bla_{IMP-13} and bla_{IMP-1} genes[a]

β-Lactam	MIC (μg/ml) for *E. coli* strain		
	DH5α(pLBII-IMP-13)	DH5α(pLBII-IMP-1)	DH5α(pBC-SK)
Ampicillin	8	32	0.5
Piperacillin	≤2	≤2	≤2
Cefoxitin	>32	>32	8
Cefotaxime	4	8	0.25
Ceftazidime	16	32	0.25
Imipenem	2	0.5	0.25
Meropenem	0.125	2	≤0.015
Ertapenem	0.125	2	≤0.015

[a] The MICs for the control strain carrying the empty vector are shown for comparison.

The analysis of the *in vitro* susceptibility of *E. coli* DH5α carrying the cloned bla_{IMP-13} gene (Table 2) was overall concordant with that of the kinetic parameters, except for piperacillin, whose MIC values remained low, likely reflecting the importance of other factors. The higher turnover rate of imipenem than of meropenem observed with IMP-13 is reflected by the MIC values of carbapenems with *E. coli* DH5α producing the IMP-13 enzyme. In addition, the MIC value for imipenem was also higher for this strain than for the IMP-1-producing clone (Table 2), while the opposite situation (lower MIC values) is observed with meropenem and ertapenem. This is probably the most important difference between IMP-13 and the other IMP-type MBLs, since the higher turnover of IMP-13 with imipenem has a significant impact on the level of resistance to that antibiotic.

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