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Biochemical Characterization of VIM-7, a New VIM Variant Produced by a *Pseudomonas aeruginosa* Clinical Isolate from Texas

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AMENDED ABSTRACT

Objectives: *bla*$_{VIM-7}$ was the first mobile metallo-ß-lactamase described in North America. This plasmid-encoded gene was found in the *Pseudomonas aeruginosa* isolate 07-406, submitted to the CANCER Program in 2001. The enzyme produced is very divergent from the other member of the VIM-family enzymes. VIM-7 has 77% amino acid identity to VIM-1, whereas VIM-1 to VIM-6 has 89 to 99% identity. The aim of this study was determine the kinetic parameters to VIM-7.

Methods: *bla*$_{VIM-7}$ was amplified and aggregated with Nde I restriction site by PCR and primarily cloned in TOPO TA Cloning vector. The gene was sub-cloned in pET-9a expression vector and overexpressed in *Escherichia coli* BL21 (DE3). The VIM-7 protein was purified using Fast Performance Liquid Chromatography (FPLC). The integrity of the enzyme was confirmed using mass spectroscopy and N-terminal sequencing. Kinetic properties were determined with several ß-lactam substrates measuring hydrolysis rates under initial rate conditions.

Results: VIM-7 varies from VIM-1 by 31% amino acid residues at the level of the mature protein, being the most divergent VIM variant so far identified. Semi-purified VIM-7 actively hydrolyses imipenem (IMI), meropenem (MER), penicillins and cephalosporins, where the penicillins and IMI appears to be the preferential substrates. The K_m (micro Molar) values obtained with semi-purified enzyme were 17 to ampicillin, 35 to IMI and 126 to MER. VIM-7 showed no hydrolysis against aztreonam and poor rates against the serine-ß-lactamase inhibitors (clavulanic acid and tazobactam). In comparison with VIM-1 and VIM-2, VIM-7 showed remarkable differences.

Conclusions: VIM-7 is a new highly divergent VIM variant, which demonstrates significantly distinct kinetic properties from those reported for VIM-1 and VIM-2. VIM-7's kinetic profile coupled with the possibility of its dissemination in America, suggests that this enzyme will compromise therapies in this region.

INTRODUCTION

In 1999, a novel series of class B metallo-ß-lactamase was initially described in Europe. The first enzyme of the VIM-series, VIM-1, was found in a strain of *Achromobacter xylosoxidans* isolated in 1997 in Verona, Italy. The VIM-2 enzyme was described in 2000 from a *Pseudomonas aeruginosa* isolate recovered in France in 1996. Since then bacterial isolates producing VIM MßL variations have been described in other countries: VIM-1, 2 and 4 in Greece, VIM-2 in France, Poland, England and Chile and Venezuela, VIM-2 and VIM-3 in Korea and Taiwan, VIM-4 in Sweden, VIM-5 in Turkey, VIM-6 in Singapore and, more recently VIM-7 in the USA.

The VIM-7 MßL was isolated from a *P. aeruginosa* isolate 07-406 recovered in 2001 in Texas, USA and submitted to the CANCER Antimicrobial Surveillance Program. VIM-7, unlike the other enzymes of the VIM-series, is markedly divergent. VIM-7 shows 77% identity with VIM-1, whereas the other enzymes vary from each other by less than 11%. Many of the changes unique to VIM-7 were changes in amino acids possessing different functions and may well alter the biochemical properties of the enzyme. In this study, we report the biochemical characterization of the new evolutionary distinct VIM variant, VIM-7, that is the first MßL being reported in the United States.

MATERIALS AND METHODS

Construction of the expression system for VIM-7 overproduction in *E. coli*. The *bla*$_{VIM-7}$ gene was amplified from *P. aeruginosa* 07-406 by PCR with primers VIM-7FNde (5'-GGAATTCCATATGTTTCAAATTCGC-3') which added Nde I restriction site (underlined) at the 5'-end of the gene and VIM-7R (5'-ACGCCCGGTGGCCGAGTAAC-3'). Amplification was carried out under standard conditions and the PCR product was primarily cloned in TOPO TA cloning (Invitrogen, Carlsbad, CA) according to the manufacture's instructions. Plasmid preparation from one colony harboring the PCR fragment was digested with Nde I and subsequently with Not I. The 0.9 Kb fragment obtained was sub-cloned into the T7-based expression vector pET-9a originating the recombinant plasmid pET_VIM-7. The plasmid was transformed into *E. coli* BL21 (DE3).

ß-lactamase purification. Cell extract from *E. coli* BL21 harboring the pET_VIM-7 recombinant plasmid was obtained by sonication. Cell debris was removed by centrifugation and the supernatant was treated with 50% ammonium sulfate and incubated for one hour at 4ºC. The sample was centrifuged (3000 g for 40 minutes at 4ºC) and then treated with 90% solid ammonium sulfate. The protein pellet obtained after centrifugation was solubilized in 50 ml buffer A (10mM Tris, 50 µM $ZnCl_2$, pH 6.5). The sample was dialyzed and loaded onto a Q-Sepharose column (Amersham Pharmacia Biotech, Uppsala, Sweden), previously equilibrated with buffer A. The proteins were eluted with a linear NaCl gradient (0 - 1 M). Fractions containing ß-lactamase activity were pooled, concentrated and then injected onto a Superdex 75 HR 10/30 column (Amersham Pharmacia Biotech) previously equilibrated with Tris buffer containing 50 µM $ZnCl_2$ and 0.2 M NaCl. Proteins were eluted at a flow rate of 0.6 mL/min. During the purification procedure the presence of ß-lactamase activity was monitored with nitrocefin solution.

Protein eletrophoretic technique. The yielded enzyme was submitted to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in order to confirm the purity.

Kinetic measurements. Reactions were performed at 20ºC with 20µl of enzyme in 1 ml of Tris buffer containing 50 µM $ZnCl_2$ and 0.2 M NaCl (pH 7.0). Hydrolysis was measured by observing the changes in absorbance due to the opening of the ß-lactam ring at a range of concentrations in a Lambda 35 spectrophotometer (Perkin-Elmer, Cambridge, UK). The steady-state kinetic parameters K_m (µM) and k_{cat} (seconds^{-1}) were deduced from the initial rates of hydrolysis by using the Hanes-Woolf plot. The extinction coefficients and wavelengths for each antimicrobial agent evaluated were those described previously.

COMMENTS

- VIM-7 polypeptide migrates with a molecular mass of approximately 28 kDa and shows an analytical pI of 5.7.
- The amino acid sequence of VIM-7 shows the lowest identity among the VIM-serie enzymes, ranging from 77.8% of homology with VIM-3 and 6 to 81.2% with VIM-4 (Figure 1).

COMMENTS (Continued)

- VIM-7 possesses the major consensus features of the MßL family: the zinc-binding motif, HXHXD, (residues 116 to 120) and the other residues involved in the coordination of the Zn^{+2} ions: Hys196, Cys221 and Hys263 (Figure 2).
- VIM-7 shows preferences for the penicillins and carbapenems (especially meropenem) among the antimicrobial agents tested (Table 1).
- The purified VIM-7 does not demonstrate hydrolytic activity against cefoxitin, ceftazidime, cefepime and aztreonam. Even after prolonged incubation (1-3 h) of the enzyme with cefoxitin and aztreonam, enzyme activity was undetectable.
- The highest k_{cat}/K_m ratios were observed with nitrocefin and penicillin (2.406 and 1.386 µM^{-1}s^{-1}, respectively). The lowest values were demonstrated with imipenem, cephalotin and cefuroxime (0.065, 0.082 and 0.096 µM^{-1}s^{-1}, respectively).
- The ratios k_{cat}/K_m of VIM-7 against most of the penicillins (carbenicillin, 0.172 µM^{-1}s^{-1}, ampicillin, 0.134 µM^{-1}s^{-1} and piperacillin, 0.198 µM^{-1}s^{-1}) are homogeneous, except for penicillin, which is ten-fold higher (1.386 µM^{-1}s^{-1}).
- VIM-7 shows markedly differences in kinetic values when compared to VIM-1 and VIM-2. In general, the kinetic parameters of VIM-7 are lower than for the other VIM-variants due to both lower K_m and k_{cat} values.

Table 1. Steady-state kinetic parameters of the purified VIM-7 in comparison with those of the other VIM-variants, VIM-1 and VIM-2.

Antibiotic	VIM-7			VIM-1			VIM-2		
	k_{cat} (s^{-1})	K_m (µM)	k_{cat}/K_m (M^{-1}·s^{-1})	k_{cat} (s^{-1})	K_m (µM)	k_{cat}/K_m (M^{-1}·s^{-1})	k_{cat} (s^{-1})	K_m (µM)	k_{cat}/K_m (M^{-1}·s^{-1})
Penicillin	20.8	15	1.386	30	840	0.036	280	70	4
Ampicillin	11	82	0.134	35	920	0.038	125	90	1.4
Carbenicillin	16.4	95	0.172	170	75	2.3	185	205	0.9
Piperacillin	17.5	88	0.198	1900	3500	0.54	300	125	2.4
Cephalothin	5.5	67	0.082	280	55	5.1	130	11	12
Cefoxitin	ND[a]	ND[a]	ND[a]	26	130	0.2	15	13	1.2
Cefuroxime	0.5	5.2	0.0961	325	42	7.7	8	20	0.4
Cefotaxime	1.9	12	0.1583	170	250	0.68	70	12	5.8
Ceftazidime	<0.01	>1000	<0.0001	60	800	0.075	3.6	72	0.05
Cefepime	<0.01	>1000	<0.0001	550	150	3.7	>40	>400	0.1
Nitrocefin	36.1	15	2.406	95	17	5.6	770	18	43
Imipenem	5.1	78	0.065	0.2	1.5	0.13	34	9	3.8
Meropenem	1.94	15	0.129	13	50	0.26	5	2	2.5
Aztreonam	ND[a]	ND[a]	ND[a]	<0.01	>1000	<0.0001	<0.01	>1000	<0.0001

a. ND = data could not be determined

Figure 1. Phylogenetic tree of the VIM-variants showing the divergence of VIM-7 in comparison with the other VIM-type enzymes. The alignment used was performed with the amino acids sequences available in the EMBL database (http://www.ebi.ac.uk/fasta33/) with CLUSTAL W method.

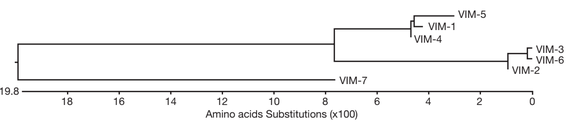
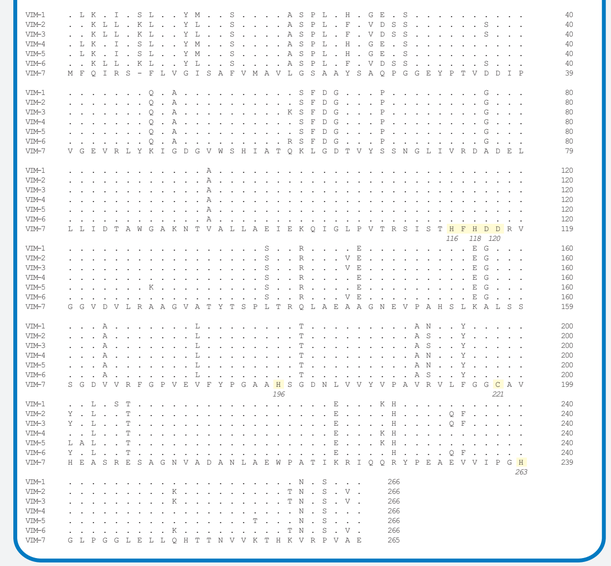


Figure 2. Amino acid alignment of the VIM-7 protein sequence with those of VIM-types enzymes. Differences in the amino acid sequences are noted by insertion of a single letter representing the amino acid change within that particular sequence. The motifs involved in the ß-lactamase activity and coordination of zinc ions are highlighted in yellow and numbered in italic according to the BBL scheme.



CONCLUSIONS

- VIM-7 activity against cephalosporins is variable, but in general it shows poor activities against most of the cephalosporins, with quite low values or no detectable activity against this group of compounds.
- Overall, the VIM-7 cephalosporinase activity is lower than those observed for VIM-1 and VIM-2.
- Structure/function studies are currently being undertaken to elucidate the hydrolytic differences observed between VIM-7 and, VIM-1 and VIM-2.

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