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# Biochemical characterization of KHM-2, a novel subclass B1 metallo-β-lactamase

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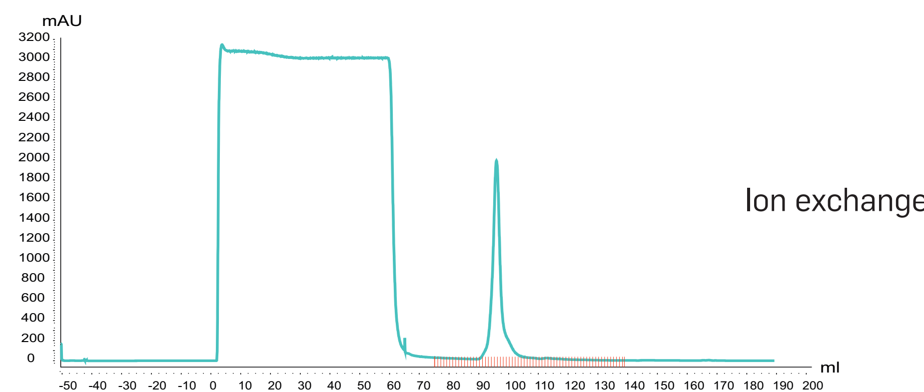
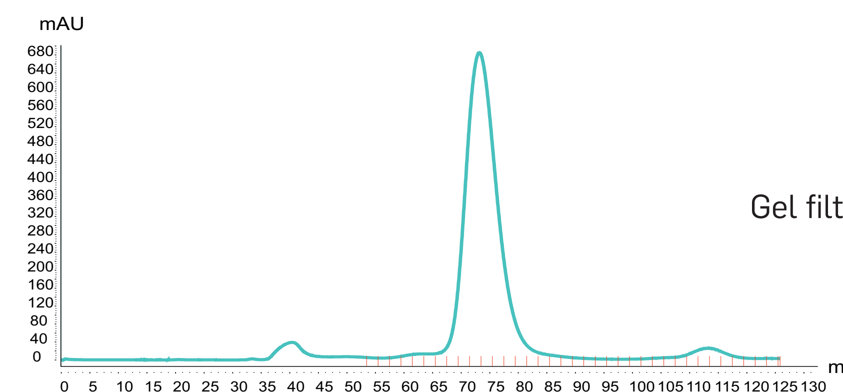
## Introduction

The worldwide increase of multi-drug-resistance in Gram-negative bacteria has become an important clinical challenge. Especially the spread of carbapenemases throughout many Gram-negative species of clinical importance is a worrying development. Carbapenemases are found in the molecular β-lactamase classes A, B and D (according to Ambler). Class B is represented by the zinc-dependent metallo-β-lactamases (MBL). MBLs are characterized by their highly efficient hydrolysis of carbapenems and almost all other β-lactam antibiotics and their insuceptibility to all clinically available β-lactamase inhibitors like sulbactam or avibactam. In 2013, a carbapenem-resistant clinical *P. aeruginosa* isolate was referred to the National Reference Laboratory for Multidrug-resistant Gram-negative Bacteria from Northern Germany. This isolate harboured the novel $bla_{KHM-2}$ β-lactamase gene, coding for a subclass B1 MBL enzyme. Here we present the purification and biochemical characterization of KHM-2.

## Purification of KHM-2




KHM-2 eluted as a single peak during ion exchange chromatography. The fractions containing the highest activity against the chromogenic cephalosporin nitrocefin were pooled and subjected to gel filtration chromatography, where KHM-2 also eluted as a single peak. With this procedure, KHM-2 and KHM-1 were purified near homogeneity with purities of up to 99 %.

## Kinetic parameters of KHM-2 in comparison to KHM-1

| | KHM-2 | | | KHM-1 | | |
|---|---|---|---|---|---|---|
| Substrate | $k_{cat}$ ($s^{-1}$)[a] | $K_m$ (µM)[a] | $k_{cat}/K_m$ ($µM^{-1} \cdot s^{-1}$) | $k_{cat}$ ($s^{-1}$) | $K_m$ (µM) | $k_{cat}/K_m$ ($µM^{-1} \cdot s^{-1}$) |
| Penicillin G | 2,101 ± 133 | 1,167 ± 222 | 1.8 | 537 ± 63 | 443 ± 164 | 1.2 |
| Ampicillin | 385 ± 27 | 683 ± 98 | 0.6 | 198 ± 23 | 1,064 ± 174 | 0.2 |
| Piperacillin | 9.9 ± 2.2 | 3,072 ± 889 | 0.003 | 18 ± 2.1 | 1,136 ± 160 | 0.0016 |
| Cefoxitin | 93 ± 3.9 | 9.8 ± 0.7 | 9.5 | 81 ± 8.5 | 7.7 ± 1.9 | 10.5 |
| Ceftazidime | 221 ± 26 | 51 ± 3.2 | 4.3 | 105 ± 14 | 66 ± 9.6 | 1.6 |
| Cefotaxime | 8.1 ± 1.0 | 5.6 ± 2.6 | 1.5 | 64 ± 17 | 6.0 ± 1.5 | 11 |
| Imipenem | 264 ± 26 | 52 ± 4.2 | 5.1 | 173 ± 49 | 66 ± 16 | 2.6 |
| Meropenem | 2.6 ± 0.4 | 3.7 ± 0.3 | 0.7 | 1.6 ± 0.2 | 1.2 ± 0.4 | 1.3 |
| Ertapenem | 2.9 ± 0.1 | 4.1 ± 0.5 | 0.7 | 1.8 ± 0.1 | 1.4 ± 0.2 | 1.3 |
| Aztreonam | NH[b] | NH | - | NH | NH | - |

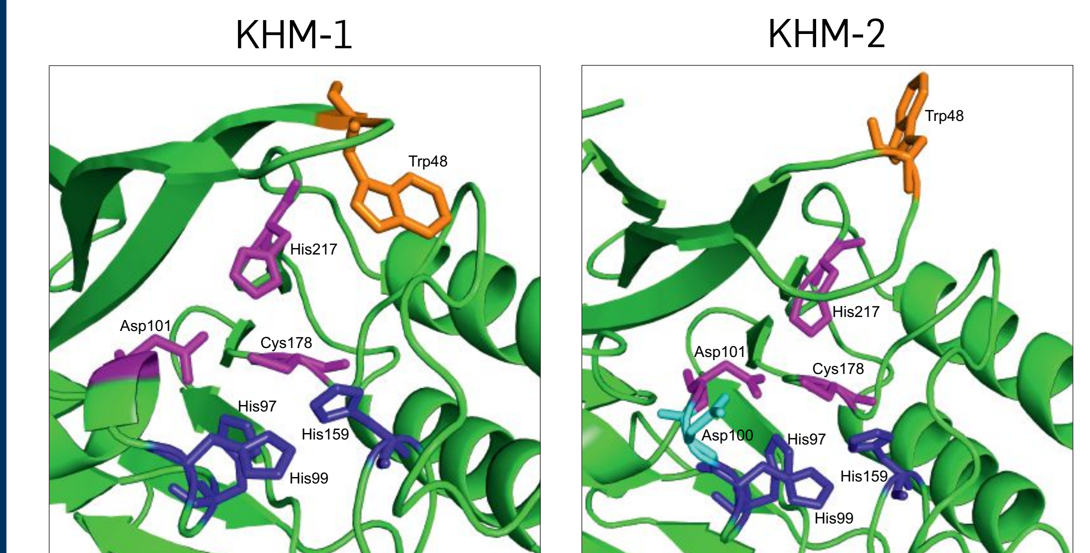
[a] $k_{cat}$ and $K_m$ values represent the means of three independent experiments with three different enzyme preparations ± standard deviations.
[b] NH, no hydrolysis was detected with a substrate concentration of up to 1 mM and an enzyme concentration of up to 200 nM.

Biochemical analysis of KHM-2 showed that the enzyme was able to hydrolyze almost all tested β-lactam substrates. Penicillin G and ampicillin were hydrolyzed with high turnover numbers, but with relatively low binding affinity, resulting in lower hydrolytic efficiencies. KHM-2 showed a very weak piperacillin hydrolysis as it is common for subclass B1 enzymes. Most cephalosporins were hydrolyzed with high efficiencies, especially cefoxitin and ceftazidime. Regarding carbapenems, KHM-2 showed the highest hydrolysis rate for imipenem, while meropenem and ertapenem were rather poor substrates with high affinity to the enzyme but with low turnover numbers. In comparison to KHM-1, KHM-2 showed higher rates for penicillins, ceftazidime and imipenem, but lower rates for cefotaxime, meropenem and ertapenem. Like KHM-2, KHM-1 showed low hydrolysis rates for piperacillin. Both KHM-2 and KHM-1 were not able to hydrolyze aztreonam, which is a common characteristic of metallo-β-lactamases.

## Methods

The KHM-2 encoding gene was cloned into the pBK-CMV vector and expressed in *E. coli* TOP10. The cells were lyzed by sonication and the cleared lysate was desalted. KHM-2 was purified by FPLC by ion exchange chromatography using a 5 ml HiTrap SP HP ion exchange column (GE Healthcare), followed by gel filtration through a HiPrep 16/60 S-200 HR gel filtration column (GE Healthcare). Hydrolysis of β-lactam antibiotics was monitored by measuring the absorbance changes resulting from the opening of the β-lactam ring. The kinetic parameters $K_m$ and $k_{cat}$ were determined by nonlinear regression using the Michaelis-Menten equation. To serve as a reference, the KHM-1 enzyme was purified and characterized the same way. Molecular modelling was performed using the SWISS-Model server.

## Homology modelling of KHM-2 and KHM-1




To gather more information on the putative influences of the mutations, the structures of KHM-2 and KHM-1 were modelled based on homologies with the crystal structure of IMP-1. Modelling showed that the zinc binding site of KHM-2 could be influenced by the T100D substitution (colored in cyan), as the zinc binding sites of both aspartic acid residues are oriented in the same direction. This could lead to disturbances in zinc coordination and to altered hydrolytic characteristics. Regarding the conserved tryptophan of the flexible loop of subclass B1 enzymes (colored in orange), the models showed that the mutations of the surrounding residues in KHM-2 lead to a conformational change of the loop, resulting in an increased distance of the tryptophan to the active site. Mutations of this residue can affect the $k_{cat}$ values. Consequently, the increased distance to the active site in KHM-2 could influence the hydrolytic characteristics of KHM-2.

## Conclusion

By characterization of the catalytic properties of KHM-2, it was shown that this novel metallo-β-lactamase was able to efficiently hydrolyze penicillins, cephalosporins and carbapenems, especially imipenem. The catalytic profile showed significant differences to KHM-1, which could be the result of an altered tertiary structure, as indicated by homology modelling.
In conlusion, the biochemical characterization of KHM-2 and the comparison to KHM-1 further underline the diversification of subclass B1 metallo-β-lactamases and the resulting differences in catalytic behaviour between enzymes of the same group. The kinetic data for KHM-2 suggest that this enzyme is a potent carbapenemase and that it most likely can confer high carbapenem resistance levels in Gram-negative species of clinical importance.

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