Institute of Pharmacology

Hannover Medical School

Viral phosphodiesterases circumvent bacterial phage defense – A characterization of the cyclic nucleotide-cleaving PDE Apyc1

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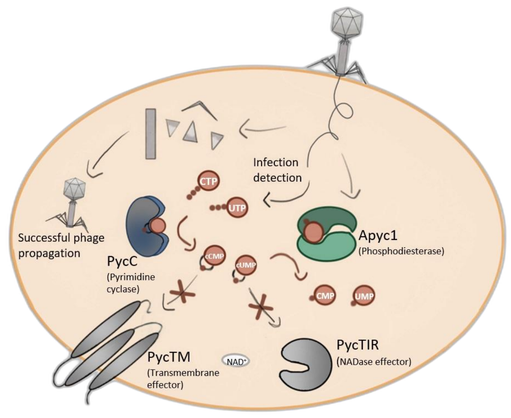
Introduction

Multi-drug-resistant bacteria pose a growing threat to the health of the population. Therefore, new antibacterial drugs are urgently needed [1]. This work deals with the characterization of a novel viral phosphodiesterase (Apyc1) as a potential antibacterial drug candidate. In nature, Apyc1 enzymes play a key role in the infection of bacteria by phages. To defend themselves, bacteria have developed various anti-phage defense systems. The system Pycsar relies on the activation of host immunity by formation of pyrimidine cyclic nucleotides (3',5'-cCMP and 3',5'-cUMP) [2]. Due to evolutionary pressure, phages, on the other hand, have developed mechanisms to circumvent bacterial immunity. Phage phosphodiesterases (PDEs) with a relaxed cyclic nucleotide specificity play a central role in disrupting the host immunity of bacteria. These PDEs are able to prevent the activation of Pycsar by cleaving 3',5'-cUMP and 3',5'-cCMP and are therefore referred to as anti-Pycsar 1 enzymes (Apyc1) [3].



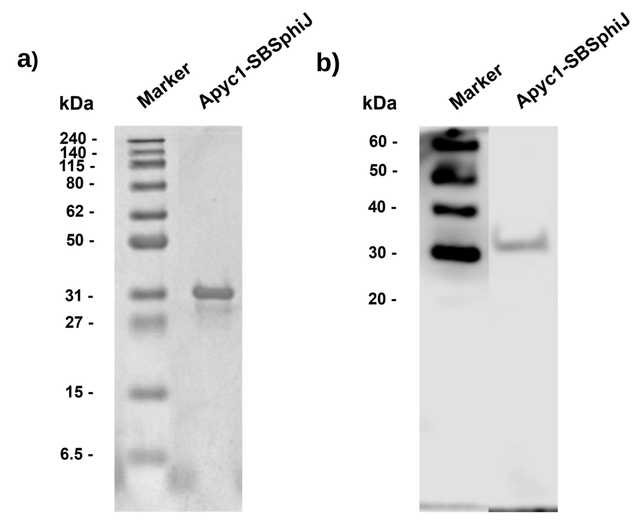
Scheme based on Hobbs et al. [3].

Methods

Apyc1 gene from Bacillus phage SBSphiJ was cloned into a pET expression vector containing an N-terminal 6x His-tag. After production in *E. coli* BL21(DE3)pLysS, Apyc1 was purified via immobilized metal affinity chromatography and size exclusion chromatography. SDS-PAGE and western blot were used for identification and purity determination. The purified enzyme was then analyzed in enzyme assays containing 3',5'-cNMPs and 2',3'-cNMPs as substrates. HEPES buffer (50 mM, pH 7.5) containing 150 mM NaCl, 1 mM DTT, 5 mM $MgCl_2$, 1 mM $MnCl_2$ and 100 µM of each 3',5'- or 2',3'-cNMP was used as a reaction buffer. Varying reaction buffer compositions were used for the DoE, which are described in the results section. The products of these reactions were unequivocally identified via LC-IMS-QTOF and quantified via LC-QQQ.

Results

Production and Purification of Apyc1

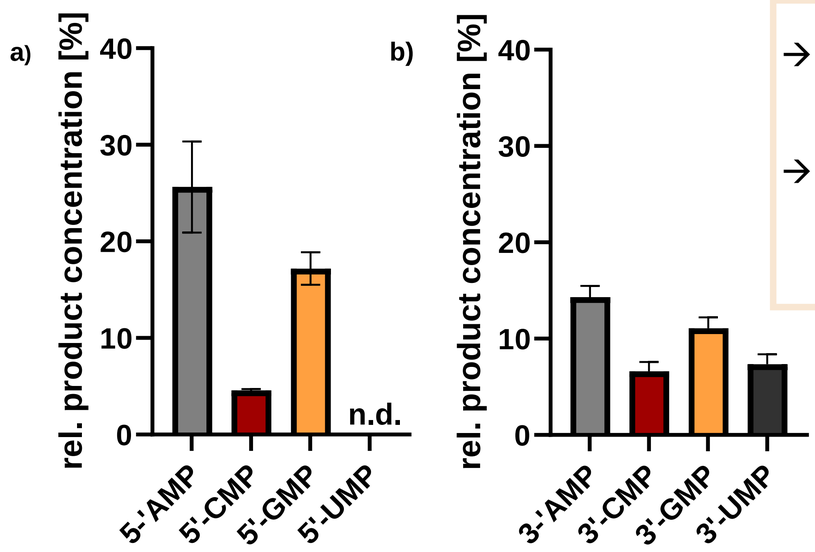


→ Detected bands fit the size of the fusion protein (32.74 kDa).

→ Enzyme purity: 88.4%

Fig. 1: SDS-PAGE analysis (**a**) and western blot (**b**) of Apyc1-SBSphiJ after affinity and size exclusion chromatography. Staining after SDS-PAGE was performed using a coomassie R-250 dye-based reagent. An anti-His-tag primary antibody and an HRP-conjugated anti-mouse secondary antibody were used for the immunoblot. Visualization of the secondary antibody was performed via chemiluminescence. The purifity of the enzyme was determined after SDS-PAGE by image evaluation via ImageJ.

Hydrolysis of 3',5'-cNMPs and 2',3'-cNMPs



→ Apyc1 hydrolyzes 3',5'-cNMPs as well as 2',3'-cNMPs.

→ Purine cyclic nucleotides were hydrolyzed faster than pyrimidine cyclic nucleotides.

Fig. 2: Enzyme assays with purified Apyc1-SBSphiJ (10 nM) in triplicates. Either 3',5'-cNMPs (**a**) or 2',3'-cNMPs (**b**) were used as substrates. Relative product concentrations (5'-AMP, 5'-GMP, 5'-UMP, 5'-CMP and 3'-AMP, 3'-GMP, 3'-UMP, 3'-CMP) are presented on the y-axis.

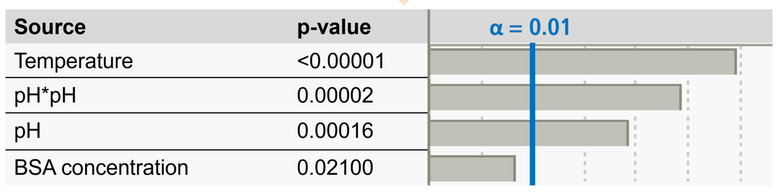
Optimization of Enzyme Assay Conditions

The effect of six different factors on the enzyme activity (hydrolysis of cGMP) was investigated using a design of experiment (DoE) approach. First, active factors were determined by a definitive screening. Levels for the active factors were then adjusted and their effect on the enzyme activity was investigated in more detail using a central composite design (CCD). Finally, the data obtained from the CCD were used to generate a predictive response surface model, allowing the identification of optimal assay conditions for subsequent kinetic characterization of the enzyme.

Screening DoE: Definitive Screening Design

Screening of six factors at three (continuous factors) or two levels (categorical factors).

Factors:
Temperature (20, 30, 40 °C); pH (5.5, 7, 8.5); BSA concentration (0, 5, 10 mg/mL)
DTT concentration (1, 5.5, 10 mM); Salt (NaCl, KCl); Salt concentration (50, 275, 500 mM)



Active factors:
Temperature
pH
(BSA concentration)

Fig. 3: Pareto plot of standardized effects of the Definitive Screening (α=0.01).

Optimization DoE: Central Composite Design

Testing of three levels including two axial points of each factor with a CCD (five center points).

Factors:
Temperature (18.2, 25, 35, 45, 51.8 °C); pH (4.5, 5.5; 7, 8.5, 9.5); BSA concentration (1.3, 4, 8, 12, 14.7 mg/mL)

The model created from the CCD Experiment included only significant terms (Temperature; Temperature* Temperature; pH; pH*pH). The model was evaluated and used for creating a response surface plot.

Tab. 1: Evaluation of the response surface model.

Key metrics	value
R^2	0.936
R^2 Adjusted	0.920
Root Mean Square Error	5.08
Mean of Response	27.68
p-value; Model	<0.001
p-value; Lack of fit	0.167
Maximal desirability	pH = 7.56; Temp. > 51.8 °C

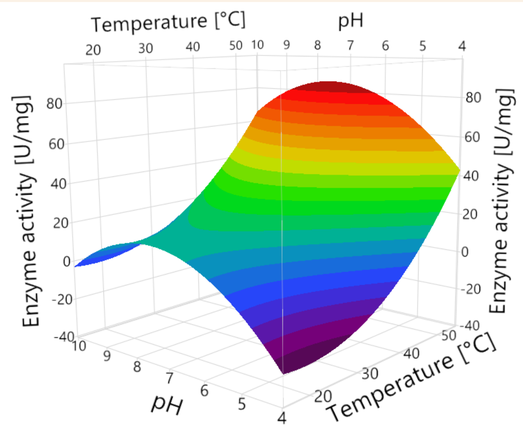


Fig. 4: Response Surface Plot of the predicted enzyme activity plotted against pH and temperature.

Conclusion and Outlook:

- Apyc1 from *Bacillus* phage Bsp38 and SBSphiJ were successfully produced and purified.
- Apyc1-SBSPhiJ exhibits hydrolytic activity toward both 3',5'-cNMPs and 2',3'-cNMPs.
- Enzyme assay conditions were optimized by DoE; Optimal temperature range was found to surpass physiological conditions.
- Future work will focus on determining the temperature optimum of Apyc1, kinetic characterization, and identifying modulators of its activity.

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[2] Tal *et al.* (2021). *Cell*, *184*(23), 5728-5739.e16.
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Metabolomics

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