Bericht zur Max-Buchner-Forschungsarbeit

Enzymatic Synthesis of Pharmaceutically Active Peptides

(MBFSt-Kennziffer: 3781)

Dr. Ayad Dawood, Institute of Technical Biocatalysis, Hamburg University of Technology

1. Aufgabenstellung und Zielsetzung

Peptides show a widespread biological activity and applicability especially in medicine.[1-3] The majority of industrial peptide production still relies on chemical synthesis and therefore the utilization of protection chemistry which makes this approach a wasteful, expensive and inefficient synthesis strategy.[4,5] So, competitive biocatalytic alternatives to chemical peptide synthesis are of great demand towards non-toxic, waste-free and chemoselective routes to target peptides.[2] *ATP-grasp* enzymes, named after the characteristic non-classical ATP-grasping folding domain, bear a great potential for enzymatic peptide synthesis. This enzyme group catalyze peptide bond formation tRNA-independently by ATP-dependent monomer activation (figure 1).[1,6,7] Additionally, these enzymes show a very diverse substrate scope, in particular regarding non-canonical amino acids.[1,6] Therefore, these enzymes could overcome the major limitations of existing biocatalytic approaches, *e.g.* the ribosomal and non-ribosomal peptide synthesis or enzymatic reactions already established in industry.[2,4,8]

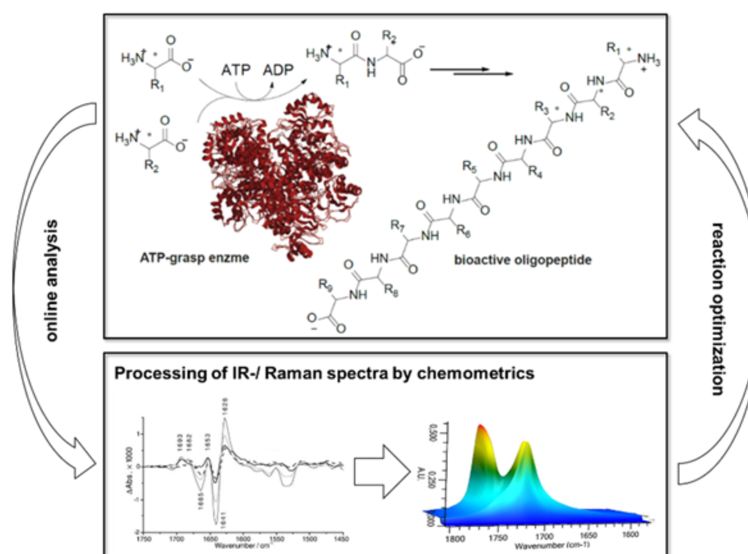

Figure 1. Schematic illustration of the project goals. Central point is the enzymatic synthesis of target bioactive and/or pharmaceutically peptides using several different *ATP-grasp* enzymes in a cascade fashion (upper box). Monomer activation takes place ATP-dependently at the carboxylic acid moiety prior attack of an amine nucleophile and therefore amide bond formation. Secondly, the establishment of an inline analysis on basis of IR or Raman spectroscopy and chemometrics is aimed (lower box) for *in situ* reaction characterization and optimization.

The evaluation of a novel biocatalytic access to biologically active oligopeptides is aimed in the form of an enzyme cascade using *ATP-grasp* enzymes (figure 1). Thus, antimicrobial peptides (AMP) are possible targets in this project. AMPs are low-molecular peptides with antibacterial, antiviral and anticancer activity and so bear a high potential for a therapeutic use.[9-11] According to the *Antimicrobial Peptide Database* the majority of AMPs is still isolated from natural sources.[12] In addition, as a process engineering aspect, the feasibility of IR or Raman spectroscopy shall be evaluated perspectively as inline analysis and *in situ* reaction monitoring on the

basis of chemometrics[13,14] (figure 1). This is not demonstrated yet for peptide synthesis and would provide a basis for a rapid reaction optimization approach.

2. Durchgeführter Arbeitsplan

Promising members of the *ATP-grasp* enzyme class - in terms of substrate scope - were identified via literature and data base mining, *e.g.* via https://www.rcsb.org, https://www.uniprot.org and https://www.ncbi.nlm.nih.gov. Respective sequences containing a histidine tag were ordered as synthetic genes after codon-optimization using the webtool *Optimizer*.[15] Since those sequences were purchased in cloning vectors a transfer to the pET expression system was needed by using the *fast cloning* approach[16](figure 2). For detailed PCR conditions see figure 3. Protocols for protein expression and purification via immobilized metal affinity chromatography (including SDS-PAGE) were performed as reported elsewhere[17] but modified here in terms of result optimization. For detailed information see the description of figure 3.

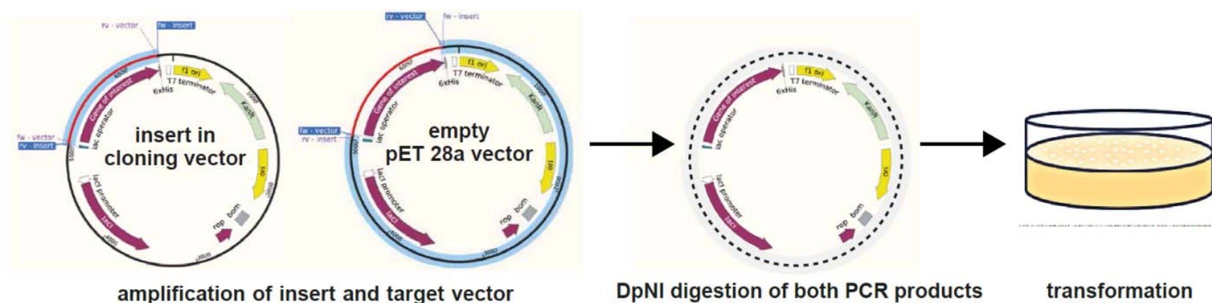

Figure 2. Schematic illustration of the *fast cloning* approach according to the protocol of Li et al.[16] Promising *ATP-grasp* enzymes were ordered as synthetic genes in a cloning vector. Primer design for each gene of interest and for the target expression vector (pET 28a) was performed according to the mentioned reference. After amplification and subsequent DpNI digestion TOP10® *E.coli* cells were transformed with a mixture of amplified insert and target vector.

3. Ergebnisse

Six promising *ATP-grasp* enzymes could be identified, cloned and purified successfully (see exemplary figure 3). Using the *fast cloning* technique all sequences could be transferred into the desired pET expression system. Protein expression occurred with high yield in the soluble fraction applying low temperatures during expression (20°C). Also, enzyme purification was performed successfully with high purity (figure 3). These enzyme preparations are supposed to be applied in UV/Vis spectroscopy for enzyme characterization (*e.g.* substrate scope and optimum in reaction conditions). Pederick et al. reported recently an optimized version of a simple, sensitive and fast method for the quantification of inorganic phosphate which is released by hydrolytic activity on ATP as a substrate.[18,19] By a molybdenum-based coordination of inorganic phosphate a blue-colored complex is formed with an absorption maximum at approx. 700 nm. To ensure a reliable screening process desired assay conditions and reaction components are needed to be investigated to exclude any interference in color formation.
However, regarding IR and Raman spectroscopy as reaction monitoring technique figure 4 demonstrates the very preliminary results obtained during this period of the project. In order to identify key regions in respective spectra glycine and its homo-oligomers (up to three monomers) where measured exemplary in *Fourier-transformed* (FT-)IR spectroscopy and Raman spectroscopy (in mixture with catalytically relevant components). Indeed, interesting regions around 1580 cm^{-1} (IR spectroscopy) and 850-950 cm^{-1} (Raman spectroscopy) were observed. But these results are needed to be verified in crude reaction mixtures and under final reaction conditions.

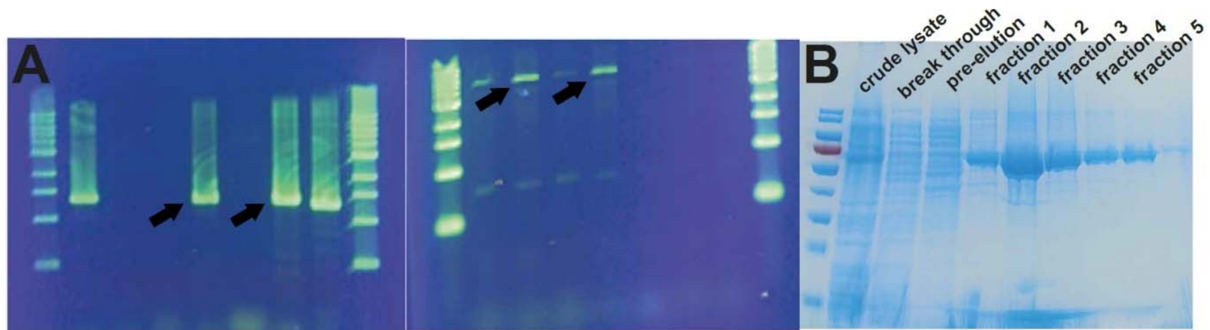

Figure 3. **A** Analysis of obtained PCR products after *fast cloning* via agarose gel electrophoresis (0.8%). The amplified gene of interest (left) and amplified target expression vector pET 28a (right) are indicated with an arrow. PCR conditions: 98 °C initial denaturation (1 min), 98 °C(10 s), 68 °C for vector or 55 °C for insert annealing temperature (10 s), 72 °C elongation temperature (50 s for vector and 26 s for insert), 19 cycles. 1 kb DNA marker was used as reference in agarose gel electrophoresis.
B Analysis of protein samples by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) after expression and purification of *ATP-grasp* enzymes. Protein expression conditions: Lysogeny broth (LB) media with 100 µg kanamycin at 160 rpm and 20°C. After the optical density at 600 nm reached 0.5–0.7 expression was induced by adding 0.2 mM isopropyl-β-D-1-thiogalactopyranoside (final concentration). After 18 h the cultures were centrifuged (4,000 x*g*, 15 min, 4 °C) and washed with lysis buffer (HEPES (50 mM pH 7.5), 20 mM $MgCl_2$, 300 mM sodium chloride). Cell disruption was performed via sonication using the Bandelin Sonoplus HD 2070 (8 min, 50% pulsed cycle, 50% power) on ice followed by centrifugation (12,000 x*g*, 45 min, 4 °C). His-Tag purification was performed as reported elsewere.[20] SDS-PAGE conditions: A 4% stacking gel and 12.5% resolving gel was used. Samples were mixed with 4-fold stock of SDS sample buffer and were denaturated by incubation at 95 °C for 10 min. Unstained protein molecular weight marker was used as reference in SDS gel electrophoresis. Protein staining was done using Coomassie Brilliant Blue®.

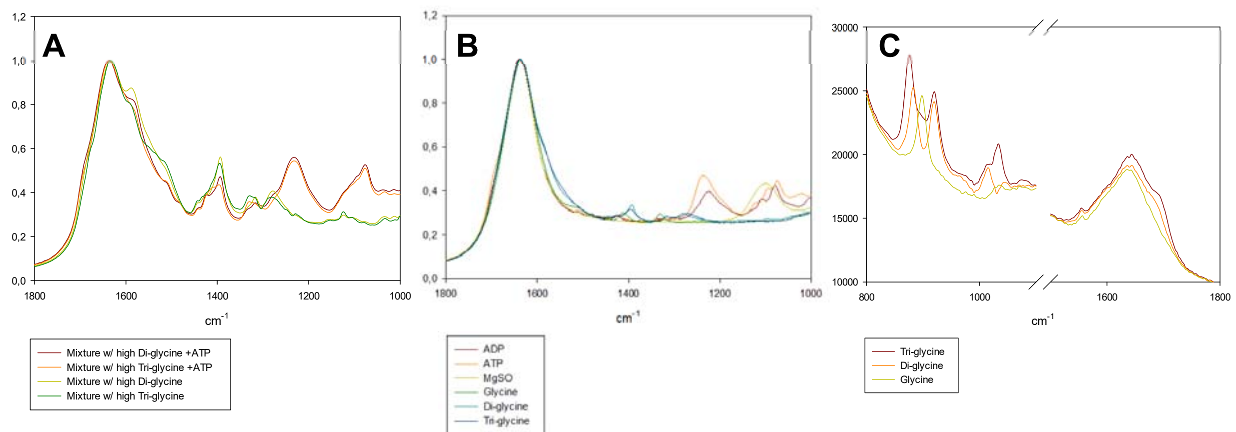

Figure 4. Spectra of FT-IR (**A** and **B**) and Raman (**C**) measurements. In case of the FT-IR measurements samples were applied onto an ATR crystal (attenuated total reflectance, see[13,14]). Glycine and its homo-oligomers (up to tri-glycine or glycylglycylglycine) were measured individually and in mixtures with various catalytically relevant chemicals: Adenosine triphosphate – ATP, Adenosine diphosphate – ADP, $MgSO_4^{2-}$. Substance concentration was 200 mM each. Significant lower concentrations resulted in sensitivity issues. Background signals were subtracted manually or by the respective software. Presumed signature regions were noticed between 1600-1500 cm^{-1} (for FT-IR, **A**) and 850-950 cm^{-1} (for Raman, **C**).

4. Fazit

As a starting point, promising *ATP-grasp* enzymes could be produced. A colorimetric assay was identified as promising and suitable for a fast *ATP-grasp* enzyme characterization. With future results from this assay available the identification of a model target (oligo)peptide and also of suitable cascade involved enzymes becomes possible in order to show a proof-of-concept of *ATP-grasp* mediated peptide synthesis in analytical scale. FT-IR or Raman spectroscopy has revealed interesting signature regions using pure solutions of reaction components. But its feasibility as a future reaction monitoring technique in biocatalytic peptide synthesis is still unknown.

5. Literatur

[1] A. Goswami, S.G. Van Lanen, *Mol. BioSyst.* **2015**, *11*, 338-353.
[2] J. Pitzer, K. Steiner, *J. Biotechnol.* **2016**, *235*, 32-46.
[3] J.S. Carey, D. Laffan, C. Thomson, M.T. Williams, *Org. Biomol. Chem* **2006**, *4*, 2337-2347.
[4] B. M. Dorr, D. E. Fuerst, *Curr. Opt. Chem. Biol.* **2018**, *43*, 127-133.
[5] V. R. Pattabiraman, J. W. Bode, *Nature* **2011**, *480*, 471-479.
[6] Y. Ogasawara, T. Dairi, *Chem. Eur. J.* **2017**, *23*, 10714-10724.
[7] M. V. Fawaz, M. E. Topper, S. M. Firestine, *Bioorg Chem.* **2011**, *39*, 185-191.
[8] T. W. Giessen, M. A. Marahiel, *FEBS Lett.* **2012**, *586*, 2065-2075.
[9] T. Baumann, J. H. Nickling, M. Bartholomae, A. Buivydas, O. P. Kuipers, N. Budisa, *Front. Microbiol.* **2017**, *8*, 124.
[10] J. Koehbach, D. J. Craik, *Trends Pharmacol Sci.* **2019**, *40*, 517-528.
[11] N. Mookherjee, M. A. Anderson, H. P. Haagsman, D. J. Davidson, *Nat. Rev. Drug Discov.* **2020**, 1–22.
[12] F. H. Waghu, S. Idicula-Thomas, *Protein Sci.* **2020**, *29*, 36–42.
[13] J. Müller, M. Neumann, P. Scholl, L. Hilterhaus, M. Eckstein, O. Thum, A. Liese, *Anal. Chem.* **2010**, *82*, 6008.
[14] J. Müller, S Baum, L. Hilterhaus, M. Eckstein, O. Thum, A. Liese, *Anal Chem.* **2011**, *83*, 9321.
[15] P. Puigbò, E. Guzmán, A. Romeu, S. Garcia-Vallvé, *Nucleic Acids Res.* **2007**, *35*, W126–W131.
[16] C. Li, A. Wen, B. Shen, J. Lu, Y. Huang, Y. Chang, *BMC Biotechnology* **2011**, *11*, 92.
[17] A. W. H. Dawood, R. O. M. A. de Souza, U. T. Bornscheuer, *ChemCatChem* **2018**, *10*, 951–955.
[18] J. L. Pederick, J. B. Bruning, *Anal. Biochem.* **2021**, *623*, 114170.
[19] G. Bartolommei, M. R. Moncelli, F. Tadini-Buoninsegni, *PLoS ONE* **2013**, *8*, e58615.
[20] A. W. H. Dawood, M. S. Weiß, C. Schulz, I. V. Pavlidis, H. Iding, R. O. M. A. de Souza, U. T. Bornscheuer, *ChemCatChem* **2018**, *10*, 3943-3949.