Progress Towards Characterization of *Bacillus subtilis* Lipase A Thermostable Mutants

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Lipase A from *Bacillus subtilis* is one of the best known lipases and a good target for examination of concepts in biocatalysis. Recently, thermostable mutants of the enzyme were obtained using B-FIT driven iterative saturation mutagenesis [1,2]. The availability of these thermostable mutants opens up the opportunity of studying the biophysical factors responsible for elevated thermostability of proteins. Here, protein sample preparation and initial characterization of both wild-type and mutant enzyme is reported.

Homogenous protein sample is required for biophysical studies. For our purpose, heterologous insoluble intracellular expression in *Escherichia coli* turned out to be a very efficient method of protein expression. The purification protocol consists of solubilization of inclusion bodies in chaotrope, subsequent re-folding followed by hydrophobic interaction and cation exchange chromatographies. The procedure typically yields 80 mg of *B. subtilis* lipase A from 1 L of culture. This protocol allows efficient isotope labeling required by protein NMR when minimal medium is used.

Uniformly ^{15}N,^{13}C- and ^{15}N-labeled thermostable mutant of lipase were prepared. 3D heteronuclear correlation spectra (HNCO, HNCACB, CBCA(CO)NH, HBHA(CO)NH, H(C)CH-TOCSY) formed the basis for resonance assignment. 3D ^{15}N- and ^{13}C-resolved [^{1}H,^{1}H]-NOESY spectra were recorded to serve as input for structure determination using CYANA [3,4]. The structure calculation is currently in progress. Dynamics studies as well as experiments using wild-type protein are to follow the NMR studies presented here.

In addition, wild-type and thermostable mutants of lipase A are being compared using biophysical tools other than NMR [5]. CD melting curves in various conditions have been recorded and thermal inactivation profiles were used to characterize lipase variants. These and other techniques will be applied to establish the basis of *Bacillus subtilis* lipase A thermostability in the future.

References:

[1] M. T. Reetz, J. D. Carballeira, A. Vogel: Iterative saturation mutagenesis on the basis of B factors as a strategy for increasing protein thermostability, *Angew. Chem. Int. Ed. Engl.*, **2006**, *45*, 7745-7751.
[2] M. T. Reetz, J. D. Carballeira: Iterative saturation mutagenesis (ISM) for rapid directed evolution of functional enzymes, *Nature Protocols*, **2007**, *2*, 891-903.
[3] P. Guntert, C. Mumenthaler, K. Wuthrich: Torsion angle dynamics for NMR structure calculation with the new program DYANA, *J. Mol. Biol.*, **1997**, 273, 283–298.
[4] T. Herrmann, P. Guntert, K. Wuthrich: Protein NMR structure determination with automated NOE assignment using the new software CANDID and the torsion angle dynamics algorithm DYANA, *J. Mol. Biol.*, **2002**, 319, 209–227.
[5] S. Ahmad, N. M. Rao: Thermally denatured state determines refolding in lipase: Mutational analysis, *Protein Sci.*, **2009**, 18, 1183–1196.