

Evolution of thermostability of a cold-active lipolytic enzyme: deep into thermal adaptation

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The biotechnological potential of cold-adapted enzymes is increasingly exploited in industrial application. The major advantages envisaged in the use of enzymes from psychrophilic organisms concern the possibility of carrying out processes at low temperature which, for example, preserve labile compound of nutritional value. However the associated thermolability is a hurdle for application in even moderate conditions or for prolonged times [2]. Our work was focussed on the lipase produced by the bacterium *Pseudomonas fragi* (PFL) which is cold-adapted and thermosensitive [1]. This enzyme was targeted by an approach of directed evolution, i.e. random mutagenesis followed by the screening for improved stability. A further constraint, equal or higher activity at low temperature allowed for selecting only variants where stability was not detrimental for protein flexibility. Two rounds of mutagenesis/selection process provided a small number of PFL variants endowed with higher stability but also with a broader temperature optimum. As a first result, we could conclude that our screening system has been able to identify mutants with both desired properties and that we effectively increased thermal stability, with minor effects on cold-adaptation. Uncoupling of cold-activity and thermolability can be of practical interest, besides providing important information about the role of specific molecular features of the protein [4]. However, detailed analyses on reaction kinetics revealed an apparent increase in efficiency when some mutants were exposed to temperatures already prohibitive for wild type. Further studies required boosting protein production through optimization of a new *in vivo* system, production of mutants bearing only selected mutations and a more detailed characterization based on inactivation kinetics, specific activity at different temperatures and structural data. Results made us conclude that temperature optimum and thermal-stability have been independently modified by different point mutations. Interestingly, several stabilizing amino acid substitutions mapped into flexible protein regions, in particular in the region known as "the lid" a surface structure that covers the active site and therefore regulate the access of substrates. These results fitted very well with previous studies of rational mutagenesis guided by the comparison with sequence and structure of homologous but more thermostable lipases [3]. To conclude, although temperature adaptation in (extremophilic) enzymes still remains difficult to predict and modify, we gained important information that will require molecular-level understanding to be fully available for practical applications.

[1] Alquati, C., et al. (2002). "The cold-active lipase of *Pseudomonas fragi*. Heterologous expression, biochemical characterization and molecular modeling." *Eur J Biochem* 269(13): 3321-8. ; [2] Cavicchioli, R., et al. (2002). "Low-temperature extremophiles and their applications." *Curr Opin Biotechnol* 13(3): 253-61. ; [3] Santarossa, G., et al. (2005). "Mutations in the "lid" region affect chain length specificity and thermostability of a *Pseudomonas fragi* lipase." *FEBS Lett* 579(11): 2383-6. ; [4] Sheridan, P. P., et al. (2000). "Approaches for deciphering the structural basis of low temperature enzyme activity." *Biochim Biophys Acta* 1543(2): 417-433.