Volume 46(1-2):25, 2002
Acta Biologica Szegediensis
http://www.sci.u-szeged.hu/ABS

DISSERTATION SUMMARY

Modification of bacterial enzymes using in vitro evolution methods

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The aim of our project is the investigation of the *in vitro* evolutionary adaptation of bacterial enzymes. The molecular basis of the heat stability of enzymes is poorly understood. The amino acid sequences and the three-dimensional structures of thermophilic enzymes and their mesophilic counterparts are usually similar. In order to change the heat-stability, many laboratories applied directed mutagenesis techniques, based on the known amino acid sequences of the homologous enzyme pairs, but the results of these experiments were not always predictable. Now we know that each thermophilic protein is stabilised by unique combinations of molecular interaction (salt bridges, hydrophobic interactions, stabilization of loops, etc.)

That is why we decided to use *in vitro* methods that mimic evolution. We chose one homologuous enzyme pair, namely HisF, from the mesophilic eubacterium *Escherichia coli* and the thermophilic archaeon *Methanococcus jannaschii*. In our experiments we randomly mutagenized the thermophilic *Methanococcus hisF* gene using error-prone PCR and a mutator strain to convert the enzyme coded by this gene into a mesophilic enzyme. We constructed recombinant plasmids, which carry the *hisF* gene of either *M. jannaschii* or *E. coli* inserted in the vector plasmid pBAD24. In these constructs the expression of the *hisF* genes are under the control of a promoter that can be regulated by the concentration of arabinose in the growth medium. The mutagenized and wild type plasmids were transformed into an *E. coli hisF*$^-$ mutant host and the transformants were grown on medium containing the inducer arabinose. The *E. coli hisF*$^-$ mutant cells expressing *M. jannaschii* HisF grew much slower than those expressing *E. coli* HisF. We have isolated a colony carrying a mutant *Methanococcus* gene variant which grew faster at 37ºC than those bacteria expressing the wild type thermophilic enzyme. In this gene we detected a missense mutation near the catalytic site. Thereafter we intended to characterize this mutant enzyme. To measure HisF activity we had to synthetize and purify its substrate, and set up the appropriate assay conditions.Having done this, currently we are in the process of determining the temperature optimum and heat stability of the mutant HisF enzyme.

The other part of our work was to engineer enzyme variants with novel catalytic properties. The *SinI* modification methyltransferase *(M.SinI*) methylates the sequence GGA/TCC. We wanted to convert the substrate specificity of *M.SinI* into the more relaxed specificity GGNCC. Our approach was to use random mutagenesis and *in vitro* DNA shuffling to isolate variants of *M.SinI* that have lost the capacity to discriminate between A/T and G/C base pairs in the center of the recognition sequence. A heterogeneous plasmid pool carrying the mutagenized and shuffled M.*Sin*I gene was digested with *Sau96*I endonuclease, an enzyme that recognizes the DNA sequence GGNCC. The selection was based upon the idea that the required M.*Sin*I mutants which have lost the ability to recognize the central A/T base pair would methylate all *Sau96*I (*i.e.* GGG/CCC and GGA/TCC) sites in the plasmid, making it resistant to *Sau96*I digestion. Recently, we successfully isolated such a *Sau96I* resistant clone. Sequencing this clone, characterization of the enzyme coded by this mutant gene, quantitative assessment of its methylation specificity is currently in progress.

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