Increased Thermal Tolerance of T. fusca β-Glucosidase via Directed Evolution

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The use of heat-tolerant enzymes can improve turnover rates and tolerance to the stresses of large-scale processes. Directed evolution with high-throughput screening was used to increase the thermal tolerance of a Family 1 β-D-glucosidase encoded by the bglC gene of the cellulolytic actinomycete Thermobifida fusca. The bglC coding sequence (provided by David Wilson, Cornell University) was mutagenized by error-prone PCR. More than 22,000 clones were picked using an Autogen Autogenesys colony picker and screened further characterized. Pair-wise combinations were then made of some of the most promising mutations. Recombinant mutant and wild type BglC proteins were purified from E. coli, and differential scanning microcalorimetry (DSC) and temperature challenge experiments were performed. One of the combinations generated a BglC protein with an increased thermal stability of at least 1°C over wild type. This protein was demonstrated to have a nine-fold increased half-life at temperatures of 62 and 64°C. Through successive rounds of mutation and screening, and additional combinations of mutations, it should be possible to further increase the thermal tolerance of this β-glucosidase.

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Summary
A secondary screen of thermal tolerance was performed on cell cultures 10 minutes over the temperature range indicated at the right of the figure. Partially de-waxed plates and challenged at high temperature (65°C). Half-lives of Protein and wild type. Color is reversed for enhanced visualization.

High-Throughput Screen
- Mutated bglC gene by error-prone PCR (~2 mutations per gene)
- Constructed expression library in pET26b+ (>100,000 clones)
- Pooled library and picked with Autogen Autogenesys system onto 384-well plates containing LB + kan + IPTG, cells grown overnight
- Made replicate arrays
- Kept plates and challenged at high temperature (65°C)
- Added X-glc and observed color development
- Picked positive clones from replicate arrays

Secondary Screen
A secondary screen of thermal tolerance was performed on cell cultures using a gradient thermocycler. Saturated IPTG induced E. coli cultures were dispersed into each column of a 96-well plate and challenged for 10 minutes over the temperature range indicated at the right of the figure. Remaining activity was assayed using X-Glc as a substrate. Clones showing thermal tolerance significantly above that of wild type in this assay (e.g., 9A, 13B, 17A, 18A, and 19A) were examined further.

Half-Life vs. Temperature
Half-lives of Com2 and wild type proteins, as determined from kinetic assays, plotted as a function of temperature. Horizontal displacement of curves demonstrates a 9°C improvement in thermal stability in Com2 over wild type. Vertical displacement of curves shows about a nine-fold increase in half-life at temperatures of 62 and 64°C.

Kinetic Assays
Kinetic assays were performed on purified proteins to assess protein activity over time at various temperatures. Comparison of Com2 to wild type is shown. PNPG was used as a substrate using protein loadings having equal activity at 50°C. Half-lives of proteins were derived from these curves.

Conclusions
- Directed evolution and high-throughput screening were used successfully to identify enhanced thermal tolerance mutations in the BglC β-glucosidase of T. fusca.
- In a screen of >22,000 clones, more than seven different mutations were identified that affected thermal tolerance.
- Combining mutations gave additive or synergistic effects.
- One mutant combination, Com2, showed an increase in thermal tolerance of more than 5°C based on activity. The half-life at temperatures of 62 and 64°C increased about nine-fold.
- Differential Scanning Calorimetry supported an increase in thermal stability of the Com2 protein.
- Emerging structural information on this β-glucosidase will shed light on the mechanism by which these mutations increase thermal stability.