Increasing Thermostability of N-carbamoyl-D-amino acid amidohydrolase by Introducing Additional Intermolecular Disulfide Bridges

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N-carbamoyl-D-amino-acid amidohydrolase is an industrial biocatalyst to hydrolyze *N*-carbamoyl D-amino acids for producing valuable D-amino acids. The crystal structure of *N*-carbamoyl-D-amino-acid amidohydrolase in the unliganded and lingaded forms demonstrate a tetramer with α-ß-ß-α fold and a C172-E47-K127 catalytic triad. Crucial binding residues N173, R175, and R176 are also identified. Four mutants were further generated to engineer enzymes with additional intermolecular disulfide bridges: P178C at helix 6, A222C at helix 8, P295C/F304C and A302C from the *C*-terminal segment near a 2-fold axis. A302C and P295C/F304C showed an increase of 8.8°C and 3.7°C respectively in apparent melting temperature than that of the wild-type enzyme, while there was hardly any change for P178C and A222C. Crystal structures of A222C and A302C were determined and showed limited conformational change. An intermolecular disulfide bridge was observed in A302C but not in A222C. Enzymatic kinetic analysis of A302C revealed a 1.5-fold enhancement in k_{cat}/ K_m at 55°C and 4.2-fold increase at 65°C. Our results suggest that introducing an intermolecular disulfide bridge at the *C*-terminal segment of *N*-carbamoyl-D-amino-acid amidohydrolase near a dyad axis is a useful approach for enhanced thermostability.

Keywords: N-carbamoyl-D-amino acid amidohydrolase, disulfide linkages, thermostabilitys