Directed evolution of N-carbamoylase for enhancement of oxidative and thermal stability

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Optically active D-amino acids are widely used as intermediate in the synthesis of antibiotics, antiviral agent, pesticides, peptide hormones and sweetener[1]. In the production process of D-amino acids developed by Yamada *et al.*, chemically synthesized 5-mono-substituted hydantoin is enantioselectively converted to N-carbamyl-D-amino acid by D-hydantoinase[2]. The intermediate is further hydrolyzed to the corresponding D-amino acid either by chemical method or by enzymatic one. Chemical decarbamoylation process gives rise to a discharge of large amount of waste, and much effort has been made to develop the enzymatic method employing N-carbamyl-D-amino acid amidohydrolase (N-carbamoylase). However, oxidative and thermal stability of N-carbamoylase was found to be low compared to D-hydantoinase, which is considered one of the limiting factors in the process development. Directed evolution of N-carbamoylase from *Agrobacterium tumefaciens* NRRL B11291 was attempted in order to simultaneously improve oxidative and thermal stability. A mutant library was generated by DNA shuffling[3], and positive clones with improved oxidative and thermal stability were screened based on the activity staining method on a solid agar plate containing pH indicator (phenol red) and substrate (N-carbamyl-D-*p*-hydroxyphenylglycine)[4]. Two rounds of directed evolution resulted in the best mutant 2S3 with a significantly improved stability. Oxidative stability of the evolved enzyme 2S3 was about 18-fold higher than wild type, and it also showed an 8- fold increased thermostability. The Km value of 2S3 was comparable to that of wild-type enzyme, but k_{cat} was slightly decreased. DNA sequence analysis revealed that six amino acid residues (Q23L, V40A, H58Y, G75S, M184L, and T262A) were substituted in 2S3. From the mutational analysis, four mutations (Q23L, H58Y, M184L, and T262A) were found to lead to an improvement of both oxidative and thermal stability. Of them, T262A had the most significant effect, and V40A and G75S only increased the oxidative stability. The positions of the mutated amino acid residues were identified in the structure of N-carbamoylase from *Agrobacterium sp.* KNK 712 and structural analysis of the stabilizing effects by each amino acid substitution was also carried out[5].

References

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