NOTES

Mutants of D-Aminopeptidase with Increased Thermal Stability

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Mutant D-aminopeptidases from Ochrobactrum anthropi with increased thermal stability were obtained by random mutagenesis. One of the mutants, no. 65, was derived from E. coli cells transformed with DNA treated with sodium nitrite. The remaining activity of the purified mutant enzyme no. 65 after heat treatment at 52°C for 10 min was 20% that of the untreated mutant enzyme no. 65, whereas the native enzyme showed 5% of the untreated native enzyme activity after the same treatment. The gene for the mutant enzyme no. 65 was sequenced and it was found that Gly155 and Gly279 in the native enzyme were replaced by Ser and Asp, respectively. Five mutants carrying one or two mutations were generated from the native gene by site-specific mutagenesis. The enhancement of the thermal stability of mutant enzyme no. 65 was attributed to the substitution of Gly155 to Ser.

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were selected from about 20,000 colonies total of E. coli transformants containing the recombinant plasmids after the mutagenesis. Two mutant enzymes originating from the E. coli cells transformed with DNA treated with sodium nitrite were found to be more resistant to the heat treatment than the native enzyme and were designated nos. 62 and 65. The mutant enzyme no. 65 appeared to be more stable than no. 62.

The mutant enzymes expressed in E. coli JM103 were purified from each transformant to homogeneity as described previously (2). The cells were harvested, suspended in 5 ml of 0.01 M KPB, pH 7.0, and disrupted for 15 min with a 20 kHz ultrasonic oscillator (UCD-130, Tosho Denki, Tokyo). The disrupted cells were removed by centrifugation at 15,000 xg for 10 min. The acrylamide concentration of the gel was 4% in the stacking gel and 10% in the separating gel. The gel was 9.0 cm high, 8.4 cm wide and 1.0 mm thick.

After heat treatment at 55°C for 10 min and centrifugation at 15,000 xg for 10 min, the mutant enzyme gave a clear band while the band of the native enzyme was very faint, suggesting that the heat-treated enzyme was not destroyed by heat treatment at 55°C for 10 min.

The activity profiles of the purified native and mutant enzymes for various heat-treatment temperatures are shown in Fig. 1. The remaining activity of the purified mutant enzyme no. 65 after the heat treatment at 50°C for 10 min was 50% that of the untreated mutant enzyme no. 65, whereas the native enzyme showed 30% of the untreated native enzyme activity after the same treatment. Similarly, after the heat treatment at 52°C for 10 min, the activity of the purified mutant enzyme no. 65 was 20% that of the untreated mutant enzyme no. 65, whereas the native enzyme showed 5% of the untreated native enzyme activity.

To investigate the effect of the heat treatment on the quaternary structure of the enzyme, the enzyme was electrophoresed before and after the heat treatment. No notable change was observed in the bands of the gel after Coomassie brilliant blue staining (0.25% CBB in methanol : acetic acid : water 5 : 1 : 5), destaining: 7% acetic acid), when the purified enzymes were electrophoresed in the absence of SDS using a vertical slab gel apparatus (DPE 2210, Daiichi Kagaku Yakuhin, Tokyo) after the heat treatment at 50°C for 10 min and centrifugation at 15,000 xg for 10 min. The acrylamide concentration of the gel was 4% in the stacking gel and 10% in the separating gel. The gel was 9.0 cm high, 8.4 cm wide and 1.0 mm thick.

After heat treatment at 55°C for 10 min and centrifugation at 15,000 xg for 10 min, the mutant enzyme gave a clear band while the band of the native enzyme was very faint, suggesting that the heat-treated enzyme was not destroyed by heat treatment at 55°C for 10 min.

As summarized in Table 1, the gene sequencing results showed that the sites of mutation in the dap gene encoding mutant enzyme no. 65 were Leu70 (CTG), Gly155 (GGC) and Gly279 (GCG), which were replaced by Leu (TGT), Ser (AGC) and Asp (GAC), respectively. One of these was a silent transition (C to T). Two other mutations were transitions of G to A. Our results are in accordance with the observations that treatment of DNA with nitrous acid results mainly in transitions of C to T and A to G, and less frequently G to A (7).

Five mutants carrying one or two mutations were generated from the native gene by site-specific mutagenesis. Three mutant plasmids with a single mutation were generated: G155S (DAP 9), G279S (DAP 10), and
G279D (DAP 11). Two more mutant plasmids with double mutations were also generated from DAP 9: G155S and G279S (DAP 12), and G155S and G279D (DAP 13). Thermal stabilities of these mutant enzymes are shown in Table 2. In the control experiment, the activity of the purified mutant enzyme no. 65 after the heat treatment at 52°C for 10 min was 20% that of the untreated mutant enzyme no. 65, whereas the native enzyme showed 3% of the untreated native enzyme activity after the same treatment. Among the mutant enzymes generated by site-specific mutagenesis, DAP 9, 12, and 13 showed higher thermal stabilities under the same conditions: they all showed 20% of the enzyme activity of the respective untreated enzymes. On the other hand, the thermal stabilities of the mutants DAP 10 and 11, which contained no mutation at amino acid position 155, were almost the same as that of the natural one: activities after the same heat treatment were 4 and 3% of the untreated native enzyme activity, respectively. Thus, the enhancement of the thermal stability of the mutant enzyme no. 65 can be attributed to the substitution of Gly155 to Ser. Presently, it is not known how this change contributes to the increase of the thermal stability.

Possibilities for future studies for improving the properties of the enzyme include random mutagenesis of the gene for the mutant enzyme no. 65 and site-specific mutagenesis at the site of Gly155 (8).

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REFERENCES