Cloning of L-Amino Acid Deaminase Gene from Proteus vulgaris

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The L-amino acid degrading enzyme gene from Proteus vulgaris was cloned and the nucleotide sequence of the enzyme gene was clarified. An open reading frame of 1,413 bp starting at an ATG methionine codon was found, which encodes a protein of 471 amino acid residues, the calculated molecular weight of which is 51,518. The amino acid sequence of P. vulgaris was 58.6% identical with the L-amino acid deaminase of P. mirabilis. A significantly conserved sequence was found around the FAD-binding sequence of flavo-proteins. The partially purified wild and recombinant enzymes had the same substrate specificity for L-amino acids to form the respective keto-acids, however not for D-amino acids.

Key words: L-amino acid deaminase; gene cloning; Proteus vulgaris

D-Amino acids are useful raw materials or intermediates for the production of pharmaceuticals, agrochemicals, and food additives. Previously, we have reported a manufacturing process for D-methionine from racemate. In this process, racemic methionine was degraded asymmetrically by the action of oxidative deamination from Proteus vulgaris IAM12003 and the resultant D-methionine was purified from the reaction mixture. We have further investigated several manufacturing processes for D-amino acids using L-amino acid degrading activity. The strain was also selected as the biocatalyst for production of several D-amino acids. To characterize the L-amino acid degrading activity, we cloned the enzyme gene.

Identification and measurement of each isomer of racemic amino acids were done by HPLC using chiral separation columns, Sumichiral OA-6100 and OA-5000 (Sumika Chemical Analysis Service, Ltd., Japan).

P. vulgaris chromosomal DNA was isolated using a Genome DNA isolation kit (BIO 101 Inc., Vista, CA). A genome library was constructed by digesting the obtained DNA with HimdIII, ligation with similarly digested pUC18, and transformation of E. coli JM83. Restriction endonuclease fragments of chromosomal DNA were subcloned into pUC18, pUC19, or pBluescript SK(+), following standard procedures. Nucleotides were sequenced by the dideoxy method using an ALF Express DNA sequencer.

Among about 500 transformants screened, one with enzyme activity harbored a plasmid with a 9.8-kb insert (pLAD-H). Based on the restriction map, we constructed various subclones and compared their enzymatic activities. E. coli harboring pLAD-CS with a 1.7-kb insert showed an enzyme activity; this plasmid was used for nucleotide sequence analysis.

We sequenced the nucleotides of the insert in pLAD-CS and found an open reading frame of 1413 bp beginning with an ATG methionine codon (Fig. 1). The protein deduced from the nucleotide sequence was composed of 471 amino acid residues with a molecular weight of 51,518. Upstream from the initiation codon, there were putative −35 and −10 regions, and an inverted repeat downstream of the termination codon.

The sequence similarity search using the Swiss-Prot database with the FASTA system from National Institute of Genetics showed that the enzyme gene from P. vulgaris was similar to the amino acid deaminase (58.6% identity) from P. mirabilis(Fig. 2). Interestingly, as shown in Fig. 3, a significantly conserved sequence was found around the FAD-binding sequence in flavo-proteins including L-amino acid oxidase.

Wild-type and recombinant enzymes were purified by almost the same procedure. The washed cells (40 g wet weight) were suspended in 200 ml of 20 mM Tris-HCl buffer, pH 8.0, and disrupted with glass beads in a Dyno-Mill. The disrupted cell suspension was centrifuged to remove intact cells. After centrifugation at 8,000 rpm for 15 min, the supernatant was fractionated by ammonium sulfate at 20 to 50% saturation and the precipitate was dissolved in 20 mM Tris-HCl buffer containing 20% saturation of ammonium sulfate. The supernatant was put on a Toyopearl HW50C column. The enzyme was eluted with the buffer with a decreasing linear gradient of ammonium sulfate concentration. After we combined the active fraction, the enzyme was precipitated by 80% saturation of ammonium sulfate and desalted by dialysis with 20 mM Tris-HCl buffer, pH 8.0. The desalted enzyme was put on a DEAE-Toyopearl column. The adsorbed enzyme was eluted with an increasing linear gradient of NaCl concentration to 0.8 M. The active fractions were combined and desalted by dialysis. The native and recombinant enzymes were purified from a cell-free extract by chromatographies with about 11 and 30% recovery, respectively. However, both enzyme preparations still have some contaminants as judged by SDS-PAGE.

Wild-type and recombinant enzymes were almost the same within the experimental error. The optimum pH was 8.0, optimum temperature was 35℃, and the en-

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zyme was stable to 30°C for 30 min at pH 8.0. Table 1 shows the substrate specificity of recombinant l-amino acid deaminase toward amino acids. L-type methionine, arginine, histidine, asparagine, aspartic acid, leucine, phenylalanine, tryptophan, and tyrosine were changed easily to their keto acid derivatives. Although the rate is low, alanine, glutamine, glutamic acid, lysine, proline, and threonine were also changed. However, the enzyme was inert on glycine, L-type valine, isoleucine, and serine and absolutely to L-amino acids. The L-methionine degradation product using expressed enzyme was the same keto acid as that of P. vulgaris intact cells and L-amino acid oxidase of Crotalus adamantineus.109

In comparison with intact cells, expressed enzyme
PVAD 56-85  
PMAD 56-85  
LAA 48-77  
DAO 2-31  
MAO-A 15-43  
Mrase 100-128  
GRase 22-50  
PHBH 4-32  

shows limited activity toward L-amino acids. In our previous report, d-valine and d-isoleucine were prepared from racemate after degradation of L-isomer by intact cells. However, the expressed L-amino acid deaminase had low activity on these L-amino acids. Duerre et al. prepared two fractions of partially purified L-amino acid oxidase from P. rettgeri (Fraction

Table 1. Substrate Specificity of Recombinant L-Amino Acid Degrading Enzyme

| Amino acids | Activity | | Amino acids | Activity |
|-------------|----------|--------------------------------------------------|----------|
|             | µmol/min | (%)                                              | µmol/min | (%)                                              |
| l-methionine | 5.43      | 100                                              | l-leucine | 5.70      | 105                                            |
| l-arginine   | 1.48      | 27.3                                             | l-lysine  | 0.19      | 3.5                                            |
| l-alanine    | 0.19      | 3.9                                              | l-proline | 0.04      | 0.74                                           |
| l-asparagine | 2.37      | 43.6                                             | l-phenylalanine | 2.03 | 37.4                          |
| l-aspartic acid | 3.01 | 55.4                                          | l-histidine | 4.34 | 79.9                                          |
| l-glutamine  | 0.06      | 1.1                                              | l-tryptophan | 2.26 | 41.6                                        |
| l-glutamic acid | 0.06 | 1.1                                         | l-tyrosine | 5.04 | 92.8                                         |

For basic amino acids: column, Sumichiral OA-6100 (Sumika Chemical Analysis Service, Ltd., Japan); mobile phase, 1 mM CuSO₄ solution; detection, UV 254 nm; flow rate, 1.0 ml/min; and temperature, 40°C were used. For the other amino acids: column, Sumichiral OA-5000 (Sumika Chemical Analysis Service, Ltd., Japan); mobile phase, 2 mM CuSO₄ solution/MeOH=85:15; detection, UV 254 nm; flow rate, 1.0 ml/min and temperature, 40°C were used. Enzyme activity was defined as the amount of L-amino acid which was converted to the keto-acid derivative. RV: relative value.
I and II) and investigated the substrate specificity. Fraction I was inert on basic amino acids and Fraction II had lower activity towards branched chain amino acids. Thus it is suggested that some other oxidase or deaminase having enough activity for L-valine and L-isoleucine, is present in the cells of *P. vulgaris*.

From the genetic and enzymatic studies, one of the L-amino acid degrading enzymes from *P. vulgaris* was identified to be L-amino acid deaminase. However, further studies will be necessary to conclude that the enzyme is an oxidase or dehydrogenase.

References


