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A high-throughput and generic assay method for the determination of substrate specificities of thermophilic α-aminotransferases

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Abstract

For the determination of substrate specificities of thermophilic α-aminotransferases (AATs), a novel high-throughput assay method was developed. In this method, a thermophilic ω-aminotransferase (OAT) and a thermophilic aldehyde dehydrogenase (ALDH) are coupled to the AAT reaction. Glutamic acid is used as an amino group donor for the AAT reaction yielding 2-oxoglutalic acid. 2-Oxoglutalic acid produced by the AAT reaction is used as an amino group acceptor in the OAT reaction regenerating glutamic acid. The amino group donor of the OAT reaction is 5-aminopentanoic acid yielding pentanedioic acid semialdehyde which is oxidized by ALDH to pentanedioic acid with concomitant reduction of $NADP^+$ to NADPH. NADPH thus produced then reduces colorless tetrazolium salt into colored formazan. To construct such a reaction system, the genes for a thermophilic AAT, a thermophilic OAT and a thermophilic ALDH were cloned and expressed in *Escherichia coli*. These enzymes were subsequently purified and used to determine the activities of AAT for the synthesis of unnatural amino acids. This method allowed the clear detection of the AAT activities as it measures the increase in the absorbance on a low background absorbance reading.

Keywords: Biocatalysis; Unnatural amino acids; α-aminotransferase; ω-aminotransferase; Enzyme assay; Aldehyde dehydrogenase; Thermophilic enzyme

1. Introduction

Aminotransferases are useful for the synthesis of unnatural amino acids and optically pure amines (Shin and Kim, 1999) that have wide usages as chiral building blocks and intermediates for the synthesis of peptidomimetic pharmaceuticals (Taylor et al., 1998; Pojikov et al., 2000). We were interested in using thermophilic AATs for the synthesis of unnatural amino acids as thermophilic enzymes are generally promising in a number of commercial applications (Sonnleitner and Fiechter, 1983) largely due to their innate biochemical characteristics such as thermostability and tolerance against chemical reagents. To select AATs convenient for the synthesis of unnatural amino acids, an efficient screening method is required. However, only a few methods have so far been reported on assaying substrate specificities of AATs (Meiwes et al., 1997). In conventional assays for AATs, the amount of oxaloacetic acid formed from aspartic acid is determined in a coupled reaction with malate dehydrogenase by the decrease in absorbance at 340 nm due to the oxidation of NADPH (Karmen, 1955). The sensitivity of this assay, however, is not high because the activity can only be determined when the decrease in the absorbance is significant comparing to the initial absorbance value. Accordingly, we developed novel coupling reactions for a thermophilic AAT assay in which the increase in absorbance was measured to improve signal to noise ratio of the assay. In this report, we demonstrated that the developed method is reliable, and convenient for the high-throughput screening of thermophilic AATs.

Abbreviations: AAT, α-aminotransferase; ALDH, aldehyde dehydrogenase; OAT, ω-aminotransferase; 1-methoxy-PMS, 1-methoxy-5-methylphenazinium methyl sulfate.

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2. Materials and methods

2.1. Cloning of AAT- OAT- and aldehyde dehydrogenase (ALDH) genes

The genes for AAT [ORF ID = ST1411 (accession # = BA000023-1537)], OATs (see below) and ALDH [ST0064 (BA000023-80)] were amplified from the genomic DNAs of thermophiles, *Aeropyrum pernix* K1 (APE), *Pyrococcus horikoshii* OT3 (PH) and *Sulfolobus tokodaii* 7 (ST), by PCR with each of the appropriate primer pairs and KOD or KOD-plus polymerase (Toyobo). Cloning of the following OAT genes, PH0138 (BA000001-139), PH0782 (BA000001-806), PH1423 (BA000001-1461), PH1716 (BA000001-1762), APE0457 (BA000002-476) and APE1464 (BA000002-1518), were performed as described previously (Koma et al., 2006). Other genes were cloned as follows. The genomic DNAs were prepared using a DNeasy Tissue Kit (QIAGEN), and target genes were amplified using the following primers: for ST0064 ALDH, 5′-CCCAAAC*CATATG*AGCGAGGTCATAGAGATTAAG-3′ (forward) and 5′-CCAAC*CTCGAG*TAATAACGTTATTGCTATTAACTTATTTTC-3′ (reverse); for ST0191 OAT (BA000023-206), 5′-CCCAAAC*CATATG*AAGTTCATTCAACTTTATGGAGATAG-3′ (forward) and 5′-CCAAC*CTCGAG*AGTAATAGCTCTTTTATTTTCCGTC-3′ (reverse); for ST1411 AAT, 5′-CCCAAAC*CATATG*TTCGAAAGATTTCTTTCAAAAGACG-3′ (forward) and 5′-CCAAC*CTCGAG*TTTAAGAAAATCTAATATAGTATTTCCTAG-3′ (reverse). The underlined sequences of the primers represent restriction sites (*Nde*I: CATATG; *Xho*I: CTCGAG). The PCR conditions for KOD and KOD-plus polymerases by Robocycler (Stratagene) were as follows: 98 °C (3 min)→[98 °C (35 s)→52 °C (35 s)→74 °C (55 s)]×25 cycles→74 °C (2 min) for KOD polymerase, and 94 °C (3 min)→[94 °C (35 s)→52 °C (50 s)→68 °C (2 min)]×15 cycles→68 °C (3 min) for KOD-plus polymerase. The ST0064 ALDH gene was amplified using KOD polymerase, while the other genes were amplified using KOD-plus polymerase. Each PCR product was purified with a GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences), digested with *Nde*I and *Xho*I, and introduced between the *Nde*I and *Xho*I sites of pET21a(+) (Novagen).

The recombinant plasmids thus obtained were introduced into *Escherichia coli* JM109 (Takara). Finally, the host strain, *E. coli* Rosetta (DE3) which co-expresses the tRNAs for rare codons, AGG/AGA (arginine), CGG (arginine), AUA (isoleucine), CUA (leucine), CCC (proline) and GGA (glycine) (Novagen), was transformed with the recombinant plasmids.

2.2. Preparation of ST1411 AAT

The *E. coli* Rosetta clone harboring the ST1411 AAT gene was cultivated at 37 °C in 100 ml Luria–Bertani (LB) medium containing ampicillin (50 μg/ml) and chloramphenicol (34 μg/ml). When OD_{660} reached about 0.5, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the medium at a concentration of 1 mM, and the cells were continuously cultivated for 20 h at 25 °C. Cells were then harvested by centrifugation at 8000 ×*g* for 10 min, washed with 20 mM phosphate buffer (pH 7.5) containing 300 mM NaCl, and then suspended in 4 ml phosphate buffer (pH 7.5) containing 300 mM NaCl and 1 mM pyridoxal-5′-phosphate (PLP). Subsequently, cells were sonicated and the supernatants obtained by centrifugation at 20,000 ×*g* for 30 min at 4 °C were applied to a Protino Ni-TED 2000 column (Macherey-Nagel) to purify the desired proteins. Afterwards, imidazole was removed and the buffer was changed to 20 mM phosphate buffer (pH 7.5) containing 1 mM PLP through ultrafiltration using Ultra-15 MW30,000 (Amicon). The protein concentration was determined using the Coomassie Protein Assay Reagent (PIERCE) and adjusted to 1 mg/ml. The purity of proteins was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins thus obtained were kept at 4 °C until being used for assays.

2.3. Preparation of ST0191 OAT and ST0064 ALDH

The respective cells of *E. coli* Rosetta clones harboring the genes for OAT (ST0191) and ALDH (ST0064) were prepared from 1 l LB medium by the same method described above. Cells were washed with 50 mM phosphate buffer (pH 7.5) containing 300 mM NaCl and then suspended in 8 ml of 50 mM phosphate buffer (pH 7.5) containing 300 mM NaCl, 1 mM PLP and 25 mM imidazole. Subsequently, cells were sonicated and the supernatants were obtained after centrifugation at 20,000 ×*g* for 30 min at 4 °C. Purification was performed at 4 °C using the AKTA system (Amersham) as follows. The supernatants were first loaded onto a His-trap HP column (5 ml, Amersham) equilibrated with 50 mM phosphate buffer (pH 7.5) containing 300 mM NaCl and 25 mM imidazole. The column was washed with 20 column volumes of the equilibration buffer and then the desired enzymes were eluted with 3 column volumes of 50 mM phosphate buffer (pH 7.5) containing 300 mM NaCl and 500 mM imidazole. Subsequently, imidazole was removed from the eluted enzyme fraction and the buffer was changed to 50 mM phosphate buffer (pH 7.2) containing 1.5 M $(NH_4)_2SO_4$ by ultrafiltration using Ultra-15 MW30,000 (Amicon). The enzyme fractions were loaded onto a HiLoad 16/10 Phenyl Sepharose HP (Amersham) column equilibrated with 50 mM phosphate buffer (pH 7.2) containing 1.5 M $(NH_4)_2SO_4$, which was then washed with 2 column volumes of the same buffer. The enzymes were eluted with 20 column volumes of 50 mM phosphate buffer (pH 7.2) with a decreasing linear gradient of $(NH_4)_2SO_4$ from 1.5 M to 0 M. Subsequently, enzyme fractions were concentrated below 1 ml by ultrafiltration using Ultra-15 MW30,000 (Amicon) and then loaded onto the HiLoad 16/60 Superdex 200 pg (Amersham) column equilibrated with 20 mM phosphate buffer (pH 7.5) containing 150 mM NaCl. Elution was performed with the equilibration buffer at a flow rate of 1 ml/min and the active fractions were collected. The concentration and purity of enzymes were examined by the methods described above. The concentration of the purified OAT (ST0191) and ALDH (ST0064) were adjusted to 1 mg/ml and 10 mg/ml, respectively, and both enzymes were kept at 4 °C until being used for assays.