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High-level Expression and Purification of Recombinant 4-Aminobutyrate Aminotransferases in *Escherichia coli*

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The protein coding sequence of the 4-aminobutyrate aminotransferase was amplified by polymerase chain reaction (PCR) from a previously cloned cDNA of pig brain using a pair of primers based on the published sequence. The amplified DNA was introduced into a T7 expression vector. Recombinant 4-aminobutyrate aminotransferases were overexpressed in *Escherichia coli*. The inclusion bodies were formed when enzyme was overexpressed. The unfolded, overproduced proteins were purified by chromatography with hydroxyapatite and refolded by a sequential dialysis method. The renatured 4-aminobutyrate aminotransferase regained the catalytic activity. However, the purified mutant protein did not show the catalytic function of 4-aminobutyrate aminotransferase.

The enzyme, 4-aminobutyrate aminotransferase (GABA-T) is a key enzyme of the 4-aminobutyric acid shunt. It catalyzes the conversion of 4-aminobutyrate to succinic semialdehyde. The enzyme is located in the mitochondrial matrix and has been highly purified from pig brain (5). The holoenzyme contains 1 mole of pyridoxal-5'-phosphate (PLP) as a cofactor and appears to be a dimeric form (~100 kDa) composed of identical subunits. 4-Aminobutyrate aminotransferase is the typical PLP-dependent transaminase. The peptide bearing the cofactor PLP covalently attached to a lysine residue has been sequenced (7). The full-length cDNA encoding the pig brain GABA-T has been isolated (9) and sequenced (12). The amino acid sequence predicted from the cDNA sequence shows that the precursor protein consists of the mature enzyme of 473 amino acid residues and an amino-terminal signal peptide composed of 27 amino acids (12, 15). The precursor of 4-aminobutyrate aminotransferase isolated from brain tissues contains PLP and exhibits catalytic activity comparable to that of the mature enzyme. The presequence peptide of the precursor 4-aminobutyrate aminotransferase does not interfere with the functional properties of the mature moiety of the enzyme (4).

In order to clarify the structure-function relationships of 4-aminobutyrate aminotransferase, we have constructed recombinant 4-aminobutyrate aminotransferases using a cDNA clone from pig brain by polymerase chain reaction techniques. Lysine residue at 330 position has been identified as a PLP binding site serving as a critical cofactor for the enzyme function (7, 8). To verify the catalytic site of 4-aminobutyrate aminotransferase, lysine 330 was mutated to arginine by site-specific mutagenesis. We describe here the high-level expression and purification of the recombinant proteins. The identification of the lysine residue at 330 in the enzyme 4-aminobutyrate aminotransferase as an active site is also reported.

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Key words: 4-aminobutyrate aminotransferase, mutagenesis, overexpression, polymerase chain reaction

MATERIALS AND METHODS

Strains, Plasmids, and Proteins

The *Escherichia coli* strain HMS174 (10) and HB101 (11) have been described. The pGP1-2 and pT7-7 expression vector were kindly provided by Dr. S. Tabor (Harvard Medical School). Restriction enzymes were purchased from United States Biochemicals Corp. T4 DNA ligase and *Taq* DNA polymerase were purchased from Promega Corp.

Other Materials

Hydroxyapatite and low molecular weight standard proteins were obtained from Bio-Rad. Isopropyl-β-D-thiogalactopyranoside (IPTG) was purchased from Boeringer Mannheim. Hydroxylamine and 4-aminobutyric acid were from Kodak. Urea was from Fluka. Other chemicals were obtained from Sigma.

Construction of Recombinant 4-Aminobutyrate Aminotransferase Expression Vectors

To clone and overproduce the premature form of 4-aminobutyrate aminotransferase, its coding sequence (12) was amplified by the polymerase chain reaction (PCR) method (6) using the 5'-end primer (CGGGATCCA-TATGGCTTCCGTGTTGCTC) and the 3'-end primer

(GATCGGGGGATCCTCGGGGC) containing a *Nde*I site and a *Bam*HI site, respectively. The PCR-generated fragment was then cloned into the *Nde*I and *Bam*HI site of pT7-7. The resulting plasmid pT7-GT-1 contains the sequence for a premature form of 4-aminobutyrate aminotransferase. A clone which would contain a mature form of the enzyme which does not have the mitochondrial leader peptide was constructed by PCR amplification with the 5'-end primer (GGATGGCATATGATCAGTCAGGCCGCAG) and the 3'-end primer (GATCGGGGGATCCTCGGGGC). The PCR-generated fragment was also cloned into the *Nde*I and *Bam*HI site of pT7-7. The resulting plasmid pT7-GT-2 would encode the mature from of the enzyme (473 amino acids). *E. coli* strain HB 101 was transformed with the ligation mixtures and positive clones were selected. The inserts were confirmed by DNA sequencing as described (16).

Construction of an Active Site Mutant of 4-Aminobutyrate Aminotransferase

The putative active site lysine residue at position 330 residue was replaced with arginine by site-directed mutagenesis using a two-stage polymerase chain reaction technique (17). In the first stage PCR, the sequences (12) from 1059 to 1500 nucleotide of 4-aminobutyrate aminotransferase gene were amplified using two primers. The upstream primer (GACCTTCAGCAGGAAGATG) contained the codon sequence of the arginine (AGG) substituted for the lysine (AAG) at 330 residue and the downstream primer was GATCGGGGGATCCTCGGGGC. The resulting 441-bp fragment was used as the downstream mega-primer in the second stage PCR to amplify coding sequences from 1 to 1500 nucleotide (12) of aminobutyrate aminotransferase, using the upstream primer CGGGATCCATATGGCTTCCGTGTTGCTC. The PCR-generated fragment was then cloned into the *Nde*I and *Bam*HI site of pT7-7. The resulting clone pT7-GT-K 330R was sequenced to confirm the replacement of lysine at 330 with arginine.

Expression of 4-Aminobutyrate Aminotransferase

Recombinant pig 4-aminobutyrate aminotransferase was overproduced using a T7 RNA polymerase expression system (18, 19, 20). To express the recombinant 4-aminobutyrate aminotransferase genes *in vivo*, pT7-GT-1 and pT7-GT-2 plasmids were introduced into the HMS 174 containing pGP1-2 by the $CaCl_2$ procedure (14). pGP1-2 contains the T7 RNA polymerase gene under the control of the inducible λP_L promoter and the gene for the temperature sensitive λ repressor cI857 (Fig. 1A). Transformants were selected on LB plates containing 50 μg/ml kanamycin and 50 μg/ml ampicillin at 30°C. Colonies resistant to both inhibitors were isolated and confirmed to contain either pT7-GT-1 or pT7-GT-2 in addition to pGP1-2. Cells were grown in LB broth containing 50 μg/ml ampicillin and 50 μg/ml kanamycin at 30°C. At the cell density of 0.5 OD_{590}, the temperature of the cultures was rapidly shifted to 42°C for 15 min. Cells were allowed to grow for an additional 2 h at 37°C. Cells were harvested, washed, and frozen in liquid N_2 and stored at -80°C.

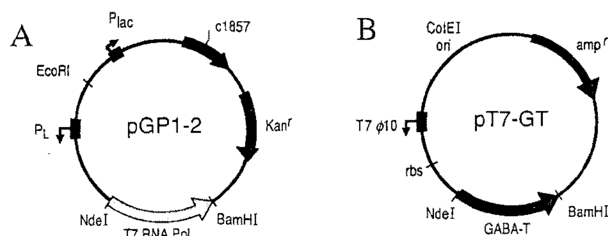

Fig. 1. Schematic diagram of the expression plasmids.
(A) pGP1-2 contains the T7 RNA polymerase gene under the control of the inducible λP_L promoter and the gene for the temperature sensitive λ repressor cI857. (B) DNA fragments containing the coding sequences for pig brain 4-aminobutyrate aminotransferases (pT7-GTs) were obtained from PCR method and ligated into T7 transcription/expression region of pT7-7 after digestion with *Nde*I and *Bam*HI.

Purification of 4-Aminobutyrate Aminotransferase

Frozen cells (2 g) were resuspended in 10 ml of 0.01 M potassium phosphate (pH 7.4), containing 1 mM α-ketoglutarate, 1 mM β-mercaptoethanol, 0.5 mM phenylmethylsulfonyl floride (PMSF), 1 mM EDTA, and 10 μM pyridoxal-5'-phosphate. After lysozyme (0.25 mg/ml) treatment, the lysed cells were sonicated with a Branson Sonifier and centrifuged in a Sorvall SS-34 rotor at 10,000×g for 30 min. The expressed proteins were precipitated into pellets. The supernatants were decanted, and the insoluble material was resuspended in 20 ml of 0.01 M potassium phosphate (pH 7.4), 1 mM DTT, and 7.2 M urea (Buffer A) to disperse the inclusion bodies. This solution was then passed over a 10 ml of hydroxyapatite column (0.9×11 cm) previously equilibrated and washed with Buffer A. The enzyme was eluted with a 100 ml linear gradient from 0.05 M potassium phosphate (pH 7.4) to 500 mM potassium phosphate (pH 7.4) containing 1 mM DTT and 7.2 M urea.

Renaturation of Purified Recombinant Pig Brain 4-Aminobutyrate Aminotransferases

Purified recombinant 4-aminobutyrate aminotransferases were renatured by a sequential dialysis method. The samples were first dialyzed against 0.01 M potassium phosphate (pH 7.4) containing 1 mM DTT and 4 M urea for at least 8 h at 4°C. The next three dialysis steps employed the same potassium phosphate (pH 7.4) containing 1 mM DTT and the following urea concentrations. The second buffer contained 2 M urea, whereas the third contained 1 M urea. The final buffer contained 0.01 M potassium phosphate (pH 7.4), 1 mM EDTA, 1 mM α-ketoglutarate, 10% glycerol, and no urea. The final dialysis was stopped after 4 h and 10 μM

of PLP was added to the dialysate. These samples were then assayed for aminotransferase activity.

Enzyme Assays

A coupled assay system consisting of 4-aminobutyrate aminotransferase and succinic semialdehyde dehydrogenase was used to study the catalytic conversion of 4-aminobutyrate to succinic semialdehyde (7). The reactions of transamination of β-alanine was determined by a fluorometric method (1).

Other Methods

Pig brain 4-aminobutyrate aminotransferase was isolated as described previously (5). Protein concentration was determined by the Bradford method (2). SDS-PAGE was performed on 12% polyacrylamide slab gels according to Laemmli (13). Proteins were transferred from polyacrylamide gels to nitrocellulose membrane electrophoretically and were immunodetected with the alkaline phosphatase system using antisera against pure 4-aminobutyrate aminotransferase.

RESULTS

Construction and Expression of 4-Aminobutyrate Aminotransferase

To overexpress the recombinant 4-aminobutyrate aminotransferases using T7 RNA polymerase expression system (19, 20). pT7-GT-1 and pT7-GT-2 were constructed as described in Materials and Methods (Fig. 1). Both premature (Fig. 2, lane 2) and mature (Fig. 2, lane 3) forms of 4-aminobutyrate aminotransferase were overexpressed representing approximately 10% of the cellular protein. Overexpression of both premature (Fig. 3, lane 2) and mature (Fig. 3, lane 3) forms of 4-aminobutyrate aminotransferase were confirmed by immunoblotting analysis using polyclonal antibody raised against native pig brain 4-aminobutyrate aminotransferase.

Purification of the Recombinant Pig Brain 4-Aminobutyrate Aminotransferase

Both premature (Fig. 2, lane 2) and mature (Fig. 2, lane 3) forms of 4-aminobutyrate aminotransferase were insoluble and formed inclusion bodies. Precipitated insoluble materials were resuspended and purified by the method described in Materials and Methods. The recombinant enzymes were retained by hydroxyapatite coumn and eluted with a linear gradient of 0.05 to 0.5 M potassium phosphate. The enzymes were eluted at 0.1 M potassium phosphate (pH 7.4), 1 mM DTT, and 7.2 M urea. Purity of the recombinant and mutant 4-aminobutyrate aminotransferases were analyzed by SDS-PAGE shown in Fig. 4. The purified recombinant 4-aminobutyrate aminotransferases were renatured by the sequential dialysis method. Both forms of the finally renatured 4-aminobutyrate aminotransferases showed activities to that of wild-type 4-aminobutyrate aminotransferase (Table 1).

Active Site Mutant of Cloned 4-Aminobutyrate Aminotransferase

The peptide carrying PLP binding site (lysine residue at 330 position deduced from cDNA) of 4-aminobutyrate aminotransferase was isolated and the amino acid se-

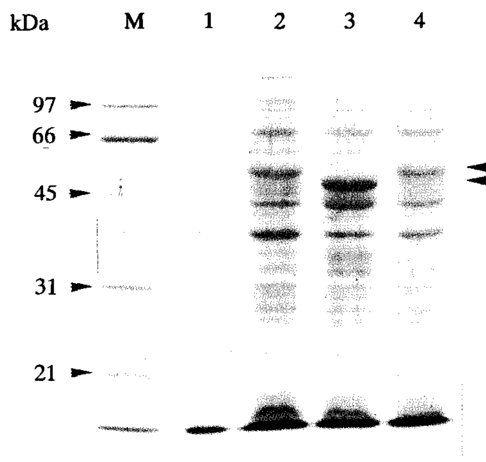

Fig. 2. Analysis of the expressed proteins by SDS-polyacrylamide gel electrophoresis.

Expressed proteins were analyzed by 12% SDS-PAGE. Lane M contains low molecular weight standard proteins; lane 1, proteins from uninduced cells; line 2, induced enzyme of premature form; lane 3, induced enzyme of the mature form; lane 4, induced enzyme of the mutated form. The gel was stained with Coomassie brilliant blue.

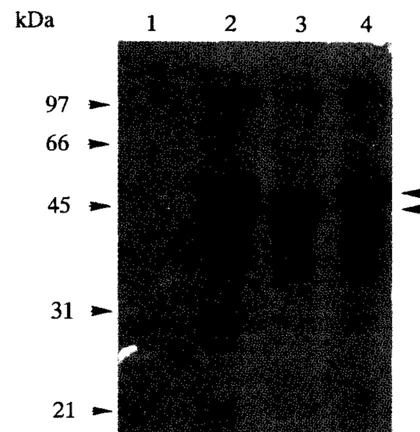

Fig. 3. Western blot analysis of the expression of recombinant 4-aminobutyrate aminotransferase.

Proteins were electrophoretically transferred from an SDS-PAGE gel to nitrocellulose membrane, probed with rabbit antisera against pig brain 4-aminobutyrate aminotransferase, and incubated with alkaline phosphatase coupled to goat antibody against rabbit IgG. Lane 1, uninduced cell extract as a control; lane 2, induced enzyme of the premature form; lane 3, induced enzyme of mature form; lane 4, induced enzyme of the mutated form. The nitrocellulose membrane was developed using the BCIP/NBT.

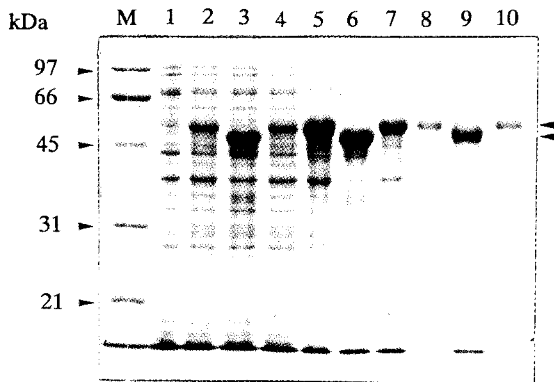

Fig. 4. Analysis of the purified recombinant 4-aminobutyrate aminotransferases.

Analysis of the purified recombinant 4-aminobutyrate aminotransferases by 12% PAGE in the presence of 0.1% SDS. Lane M, low molecular weight standard proteins; lane 1, uninduced cell extracts as a control; lane 2, induced premature enzyme; lane 3, induced mature enzyme; lane 4, induced mutant enzyme; lane 5, dissolved precipitates of premature enzyme in 7.2 M urea; lane 6, dissolved precipitates of mature enzyme in 7.2 M urea; lane 7, disslved precipitates of mutant enzyme (GT-K330R) in 7.2 M urea; lane 8, purified premature form of enzyme; lane 9, purified mature form; lane 10, purified mutant form.

Table 1. Enzymatic activities of purified and renatured 4-aminobutyrate aminotransferases.

Enzyme forms	Proteins after renaturation (mg/ml)	Specific activity at 25°C (units/mg)
mature	2.1	10
premature	1.9	9.5
K330R	0.8	0.2

4-aminobutyrate aminotransferases were purified from hydroxyapatite column chromatography. The retained enzymes were eluted with a linear gradient of 0.05 to 0.5 M phosphate buffers as described under Materials and Methods. The catalytic activity of the endogeneous enzyme in host *E. coli* HMS174 cells was determined below 0.001 unit/mg and of the wild-type enzyme from brain tissues below 12 unit/mg, respectively.

quence was determined (7, 15). In order to confirm whether the lysine residue at 330 position is critical for the catalytic function of 4-aminobutyrate aminotransferase, we have constructed the site-specific mutant (pT7-GT-K 330R) in which the lysine residue at 330 was changed to arginine using two stage polymerase chain reaction. GT-K330R mutant protein was overexpressed (Fig. 2, lane 4). Purification of GT-K330R protein was performed by the same method which was applied to the purification of premature and mature forms of enzymes. Purification of mutant 4-aminobutyrate aminotransferase is summarized in Fig. 4. Purified and renatured K330R protein did not show the catalytic activity of 4-aminobutyrate aminotransferase (Table 1). This result strongly suggests that the lysine residue at 330 is essential for the catalytic action of 4-aminobutyrate aminotransferase serving as the PLP binding site.

DISCUSSION

In this paper we have described the purification of the premature and mature forms of recombinant 4-aminobutyrate aminotransferase of pig brain from an overproducing bacterial strain. Recombinant 4-aminobutyrate aminotransferases were expressed in *E. coli* using T7 promoter and RNA polymerase expression system. The analysis of SDS-polyacrylamide gel electrophoresis of the cell extracts demonstrated that the overexpressed proteins corresponded a mature (55 kDa) and a premature (50 kDa) forms, respectively (Fig. 2 and 3). Both premature and mature forms of 4-aminobutyrate aminotransferase were well overexpressed and represented approximately 10% of the cellular proteins.

Fractionation of cell extracts by centrifugation clearly demonstrated that the expressed proteins were mostly insoluble and formed inclusion bodies. Precipitated insoluble materials were resuspended and purified. After purification, both forms of purified proteins were renatured by a sequential dialysis method, yielding catalytically active preparations. Brown *et al.* (3) evaluated for the overproduction and renaturation of the *Saccharomyces cerevisiae* recombinant DNA polymerase (3). Results indicated that the maximum renaturation yield was obtained when the ratio of dialysis buffer volume to sample volume was 50:1, with higher ratios giving poorer yields as judged by the amount of soluble fractions. Increased osmotic pressure at higher ratios may cause urea to be removed too rapidly, forcing the protein refolding into an insoluble form again (3). The specific activity of the finally renatured 4-aminobutyrate aminotransferase was almost similar to that of 4-aminobutyrate aminotransferase purified from pig brain tissues (7, 8); the overall yield from bacteria was considerably higher than from tissue and the purity was greater.

In order to verify the precursor of mitochondrial 4-aminobutyrate aminotransferase, the purified enzymes were analyzed by Western blotting. The Western blot patterns show the presence of two protein bands differing in electrophoretic mobility. The size of the precmature protein was about 55 kDa consistant with that deduced from the cDNA sequence, whereas the mature enzyme exhibits a relative molecular mass of about 50 kDa. It was shown that the signal peptide of 4-aminobutyrate aminotransferase did not interfere with the folding and functional properties of the mature moiety of the enzyme (15).

In order to clarify the molecular structure-function relationships of the target enzyme, we have constructed the site-specific mutant of 4-aminobutyrate aminotransferase. Lysine residue at 330 position is the PLP binding site which is serving as a critical cofactor for enzyme function. To verify the catalytic site of the enzyme, this lysine was replaced with arginine by site-specific mutagenesis.

The overexpression, purification, and renaturation of the active site mutant were performed by the same method applied to the premature and mature forms of the enzymes. Purified mutant protein did not show the catalytic function of 4-aminobutyrate aminotransferase. Thus, substitution of arg for lys (GT-K330R) caused the loss of enzyme activity. This result strongly suggests that the lysine residue at 330 is involved in the catalytic action of 4-aminobutyrate aminotransferase.

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