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# Synthesis of 1-Piperideine-6-carboxylic Acid Produced by L-lysine-ϵ-aminotransferase from the *Streptomyces clavuligerus* Gene Expressed in *Escherichia coli*

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The gene (*lat*) encoding L-lysine ϵ-aminotransferase (LAT) in *Streptomyces clavuligerus* was cloned and expressed in *Escherichia coli*. Nucleotide sequence analysis of *lat* predicted a single open reading frame (ORF) of 1371 bp, encoding a polypeptide of 457 amino acids with calculated molecular mass of 49.89 kDa. *S. clavuligerus* LAT was grouped into aminotransferase subfamily II of α family on the basis of sequence homology. A model system composed of the recombinant LAT in phosphate buffer was set up to study the biosynthesis of 2-acetyltetrahydropyridine. Lysine was found to be transformed to 1-piperideine-6-carboxylic acid. 2-Acetyltetrahydropyridine was characterized from the mixture of 1-piperideine-6-carboxylic acid and methylglyoxal. For the first time, we demonstrated that the L-lysine ϵ-aminotransferase is responsible for the formation of 1-piperideine-6-carboxylic acid, which may react with methylglyoxal to generate the acylated N-heterocyclic odorant 2-acetyltetrahydropyridine.

**KEYWORDS: 2-Acetyltetrahydropyridine; L-lysine ϵ-aminotransferase; methylglyoxal; *Streptomyces clavuligerus*; 1-piperideine-6-carboxylic acid; biosynthesis**

## 1. INTRODUCTION

Mousy off-flavor represents a serious form of microbiologically induced spoilage of wine. The compounds cause mousy off-flavor are the powerful odorous N-heterocyclic compounds 2-ethyltetrahydropyridine, 2-acetyl-1,4,5,6-tetrahydropyridine and 2-acetyl-1-pyrroline. Among them, 2-acetyl-1,4,5,6-tetrahydropyridine and 2-acetyl-1-pyrroline are the most potent, having odor thresholds in water of 1.6 and 0.1 μg/L, respectively (*1*). Wines affected by mousy off-flavor have been found to contain one or more of 2-ethyltetrahydropyridine (2.7−18.7 μg/L), 2-acetyl-1,4,5,6-tetrahydropyridine (up to 7.8 μg/L), and 2-acetyl-1-pyrroline (4.8−106 μg/L). Interestingly, 2-acetyl-1,4,5,6-tetrahydropyridine, which occurs in tautomeric equilibrium with its imino form 2-acetyl-3,4,5,6-tetrahydropyridine, is also a potent crackerlike flavor (*2*).

Intensive discussion about the formation of 2-acetyltetrahydropyridine was reviewed by Adams and De Kimpe (*3*). Research on thermal generation of 2-acetyltetrahydropyridine could be dated back to 1969, when Hunter et al. (*4*) characterized 2-acetyl-1,4,5,6-tetrahydropyridine as a crackerlike note and pointed out that reaction between proline and 1,3-dihydroxyacetone may lead to 2-acetyltetrahydropyridine. At present, only a limited number of synthetic routes toward 2-acetyltetrahydropyridine have been reported. Büchi and Wüest (*5*) synthesized 2-acetyl-1,4,5,6-tetrahydropyridine by hydrogenation of 2-acetylpyridine over a rhodium catalyst and oxidation of the resulting amino alcohol with a silver reagent. A straightforward synthetic route by utilizing piperidine as starting material to 2-acetyl-1,4,5,6-tetrahydropyridine was reported by De Kimpe and Stevens (*6*). Later, a reaction sequence involving deprotonation of a vicinal diimine and subsequent alkylation with an N,N-diprotected ω-bromoalkylamine, followed by deprotection and intramolecular transimination of the functionalized intermediate was developed by the same authors (*7*).

Very limited information on the biosynthesis of 2-acetyltetrahydropyridine has been reported. Biosynthesis of 2-acetyl-1,4,5,6-tetrahydropyridine in *Saccharomyces cerevisiae* during bread fermentation (*8*) has been published. Recently, heterofermentative *Lactobacillus* spp., including strains of *Lactobacillus hilgardii* and *Lactobacillus brevis*, were reported to be responsible for the production of 2-acetyl-1,4,5,6-tetrahydropyridine in a synthetic medium. Among the 34 strains of wine lactic acid bacteria, *Lactobacillus* spp., *Osenococcus oeni*, and a strain of *Leuconostoc mesenteroides* were found capable of producing mousy off-flavor in an ethanolic grape juice medium (*9*).

1-Piperideine-6-carboxylic acid (P6C) is an important intermediate in the L-lysine to L-pipecolic acid pathway for the synthesis of β-lactam antibiotics. In *S. clavuligerus*, L-lysine could be transformed by L-lysine ϵ-aminotransferase (LAT, E.C. 2.6.1.36) to form P6C (*10*). In addition to *Streptomyces clavuligerus*, lysine was converted to P6C by *Flavobacterium lutescens* (*11*), *Pseudomonas aeruginosa* (*12*), *Nocardia lac-*

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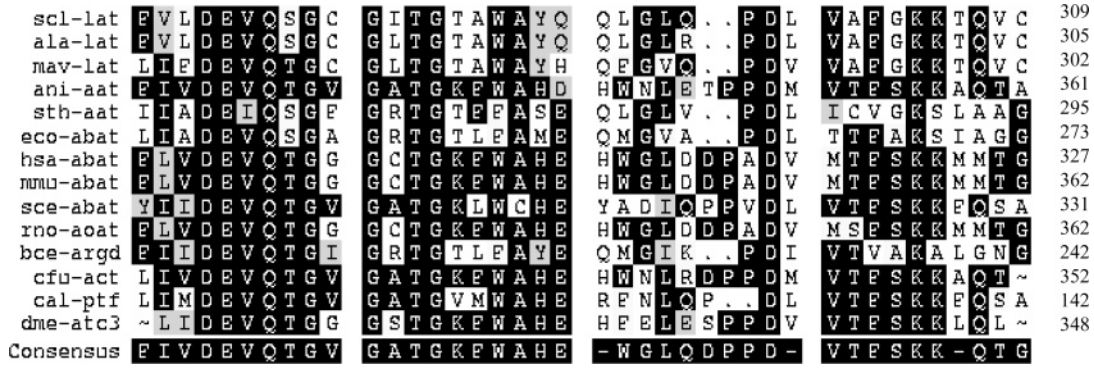


**Figure 1.** Optimal alignment of PLP attachment site of *S. clavuligerus* LAT with those of other aminotransferases. The sequences listed are those of *S. clavuligerus* lysine 6-aminotransferase, *Amycolatopsis lactamdurans* lysine 6-aminotransferase (Genbank Accession Number CAA79796), *Mycobacterium avium* subsp. *paratuberculosis* str. K10 lysine 6-aminotransferase (NP_962344), *Aspergillus nidulans* FGSC A4 gata_emeni 4-aminobutyrate aminotransferase (EAA63933), *Symbiobacterium thermophilum* IAM 14863 4-aminobutyrate aminotransferase (YP_074445), *Escherichia coli* K12 4-aminobutyrate aminotransferase (NP_417148B), *Homo sapiens* 4-aminobutyrate aminotransferase (AAD14176), *Mus musculus* 4-aminobutyrate aminotransferase (NP_766549), *Saccharomyces cerevisiae* 4-aminobutyrate aminotransferase (CAA36833), *Rattus norvegicus* $\beta$-alanine oxoglutarate aminotransferase (BAA25570), *Bacillus cereus* ATCC 10987 acetylornithine aminotransferase (NP_980493), *Cladosporium fulvum* $\gamma$-aminobutyric acid transaminase (AAG17665), *Candida albicans* SC5314 potential GABA transaminase fragment (EAL02107), *Drosophila melanogaster* aminotransferase class-III (AAM29596). The amino acid numbers are listed on the right. Boxes with black backgrounds indicate identical residues.

*tamdurans* (*13*), *Agrobacterium tumefaciens* (*14*), and *Streptomyces* spp. (*15*).

Methylglyoxal has been used as one of the major intermediates for 2-acetyltetrahydropyridine synthesis in model systems (*16*). Wiseblatt and Zoumut (*17*) reported that 1,3-dihydroxyacetone is an important intermediate in Maillard browning and the bread flavor compound can be synthesized from a reaction of proline with 1,3-dihydroxyacetone. Recently, Adams et al. (*18*) demonstrated that the bread flavor component 2-acetyl-1,4,5,6-tetrahydropyridine was formed in a proline/1,3-dihydroxyacetone model system. Methylglyoxal is predominantly generated by the nonenzymatic degradation of triose phosphate intermediate through fragmentation and removal of phosphate from the phospho-ene-diolate form of glyceraldehyde 3-phosphate and 1,3-dihydroxyacetone phosphate (*19*). Riddle and Lorenz (*20*) demonstrated that toxic levels of methylglyoxal might derive from glycerol and 1,3-dihydroxyacetone via nonenzymic reaction in Ringer's phosphate suspensions of avian spermatozoa. Significant amounts of α-dicarbonyl compounds such as methylglyoxal (*21*) and dihydroxyacetone (*22*) have been found in wine. These compounds come either from alcoholic fermentation or from the oxidation of grape sugars in wine (*23*). Ahmed et al. (*24*) pointed out that acetone, threonine, and glucose may generate methylglyoxal. Among them, formation of methylglyoxal from glucose through the triose phosphate pathway deserves special interest. Phillips and Thornalley (*19*) ascribed the formation of methylglyoxal to the excess glucose metabolized through Embden−Meyerhof pathway.

We hypothesized that methylglyoxal may react with P6C derived from the LAT to form 2-acetyltetrahydropyridine. The objective of this experiment is to provide direct evidence that LAT is responsible for 2-acetyltetrahydropyridine biosynthesis in *S. clavuligerus*.

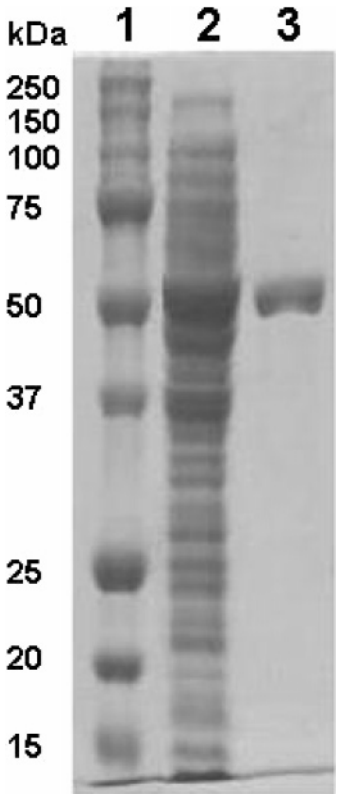


**Figure 2.** SDS–PAGE analysis of purified *S. clavuligerus* LAT. Lane 1, molecular mass standard; lane 2, crude cell extract from *E. coli* JM109/pGEM-scLAT; lane 3, recombinant LAT purified from *E. coli* JM109/pGEM-scLAT by Sephacryl S-200 chromatography. The proteins were analyzed by SDS–10% PAGE, and the gel was stained with Coomassie brilliant blue R-250.

## MATERIALS AND METHODS

**Bacterial Strains, Plasmids and Cultivation Conditions.** *Escherichia coli* JM109 and XL1-Blue (Stratagene, La Jolla, CA.) were used as hosts for recombinant plasmids. *S. clavuligerus* was cultivated at 30 °C in YM medium containing 0.4% yeast extract, 1% malt extract, and 0.4% dextrose. All *E. coli* strains were grown in Luria−Bertani (LB) medium at 37 °C. Plasmids pGEM-7Zf (+) (Promega, Madison, WI), pGEM-T easy vector (Promega, Madison, WI), and pET21b (Novagen, Madison, WI) were used as cloning and expression vectors.

**Cloning of *S. clavuligerus lat* Gene.** On the basis of the nucleotide sequence of *lat* from *S. clavuligerus* (*25*), the forward primer (5′-GAGGATCCAGGAGTTCTCACCATGG-3′; the *Bam*HI site is underlined) and reverse primer (5′-GGCGAATTCGCGTCAGACGCTC-3′; the *Eco*RI site is underlined) were designed. PCR was performed with a model PE2400 automatic thermocycler (Perkin-Elmer Cetus,

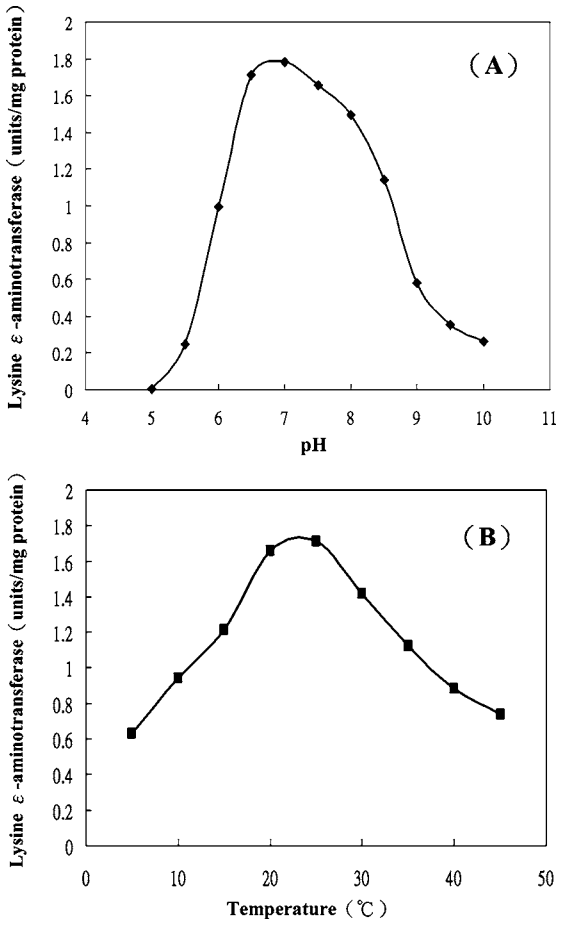


**Figure 3.** Influence of (**A**) pH and (**B**) temperature on the activity of recombinant LAT. Recombinant LAT was incubated with lysine in 1-mL buffers at various pHs and temperatures. Vertical bars indicate standard deviation.

Norwalk, CT). The 1476 bp PCR products were digested with *Bam*HI and *Eco*RI, and ligated to the pGEM-7Zf (+) vector digested with the same enzymes, generating pGEM-scLAT. The nucleotide sequence was confirmed by sequencing with an automated laser fluorescence sequencer (Model 377, ABI PRISM, Foster City, CA).

**Expression and Purification of LAT in *E. coli*.** *E. coli* JM109 containing pGEM-scLAT was grown at 37 °C (in 300 mL of LB medium with ampicillin). When the optical density at 595 nm reached 0.6−0.8, the temperature was lowered to 30 °C and protein expression was induced by the addition of 0.5 mM IPTG (isopropyl $\beta$-D-thiogalactopyranoside). Following overnight incubation, the cells were harvested and resuspended in PP buffer (0.2 M potassium phosphate buffer containing 10 $\mu$M pyridoxal 5′-phosphate, pH 7.5), and disrupted by sonication (180 W output, 30 s × three times at 0 °C). Cell debris was removed by centrifugation at 15000*g* for 15 min. Ammonium sulfate was added to the cell extract to 30% saturation. After stirring for 1 h, the precipitate was removed by centrifugation at 14000*g* for 10 min. The supernatant was adjusted to 60% saturation with ammonium sulfate to precipitate the enzyme. After the solution was stirred for 1 h, the precipitate was collected by centrifugation at 15000*g* for 15 min and dissolved in PP buffer. The solution was subjected to gel filtration on a Sephacryl S-200 column (2.6 × 60 cm; GE Healthcare, Milwaukee, WI) pre-equilibrated with PP buffer. Fractions were eluted at a flow rate of 20 mL/h in the same buffer, and the active fractions were collected. The purified enzyme was analyzed by sodium dodecyl sulfate−10% polyacrylamide gel electrophoresis (SDS−PAGE). The protein concentration was measured according to the Bradford method.

**Assay of LAT Activity.** LAT activity was determined by measuring the formation of 1-piperideine-6-carboxylate by use of *o*-aminobenzaldehyde reagent (*13*). LAT activity was assayed at 37 °C for 60 min in 2 mL of 20 mM potassium phosphate buffer (pH 7.2) containing 20 $\mu$M L-lysine, 20 $\mu$M α-ketoglutarate, and 75 nM pyridoxal 5′-phosphate. The reaction was terminated by the addition of 1 mL of 5% trichloroacetic acid (TCA). After centrifugation, the supernatant was analyzed for the amount of 1-piperideine-6-carboxylate by adding 1.5 mL of 4 mM *o*-aminobenzaldehyde. The concentration of 1-piperideine-6-carboxylate was determined by measuring the absorbance at 465 nm by use of the extinction coefficient 2800 $M^{-1}$ $cm^{-1}$ (*12*). One unit of LAT activity was defined as the amount of enzyme that liberates 1 nmol of product/min under the assay conditions used.

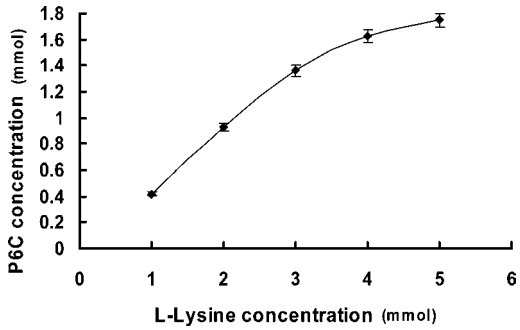


**Figure 4.** Amounts of P6C recovered correlate positively with lysine concentration.

**Synthesis of 1-Piperideine-6-carboxylic Acid and Extraction of 2-Acetyltetrahydropyridine.** For synthesis of 1-piperideine-6-carboxylic acid (P6C), purified LAT (120 units) was incubated with 1−5 mmol of L-lysine, 5 mmol of α-ketoglutarate, and 15 mmol of pyridoxal-5′-phosphate in 100 mL of potassium phosphate buffer (pH 7.2). The mixture was kept under constant agitation at 37 °C for 1 h. The recovery of P6C was determined colorimetrically with *o*-aminobenzaldehyde. The solution containing 1-piperideine-6-carboxylic acid (20 mmol) was mixed with methylglyoxal (20 mmole) in 3 mL of potassium phosphate buffer (pH 7.2). After being stirred for 2 h at room temperature, the mixture was extracted eight times with dichloromethane (total volume = 250 mL). After being dried with anhydrous sodium sulfate, the extract was further concentrated to a volume of 2 mL for GC-MS analysis.

**Gas Chromatography−Mass Spectrophotometry.** An aliquot (0.5 $\mu$L) of the extracted sample was injected into a gas chromatograph−mass spectrometer (Agilent 6890 and HP 5973 mass-selective detector, Agilent Technologies, Palo Alto, CA) equipped with a fused silica capillary column, HP-5MS, with (5% phenyl)methylpolysiloxane as nonpolar stationary phase (30 m × 0.25 mm i.d. × 0.25 $\mu$m). The sample was injected with split ratio 20:1, and the flow rate of helium was 1 mL/min. The injection port temperature was 250 °C. The column temperature program started at 35 °C upon injection. The temperature was increased at a rate of 3 °C /min to 195 °C and then at a rate of 6 °C/min to 230 °C and held for 5 min. The mass spectrometer was operated in electron impact (EI) mode with an electron energy of 70 eV; ion source temperature 250 °C; mass range *m*/*z* 35−350; scan rate 0.68 s/scan; and EM voltage 1832 V. The mass transfer line was set to 280 °C.

**Nucleotide Sequence Accession Number**. The nucleotide sequence of *lat* has been deposited with the NCBI database under Accession No. AY742798.

## RESULTS AND DICUSSION

**Nucleotide and Amino Acid Sequence of the LAT Gene.** The gene encoding LAT was obtained from *Streptomyces clavuligerus* by PCR cloning. The sequence revealed that the open reading frame (ORF) of 1371 bp encodes a polypeptide of 457 amino acids with calculated molecular mass of 49.89 kDa. LAT is the key enzyme in *S. clavuligerus* for cephamycin C biosynthesis (*26*). The *lat* genes, encoding LATs, were also cloned from *Agrobacterium tumefaciens* (*13*), *Nocardia lactamdurans*, *Mycobacterium tuberculosis*, and *Flavobacterium lutescens*.

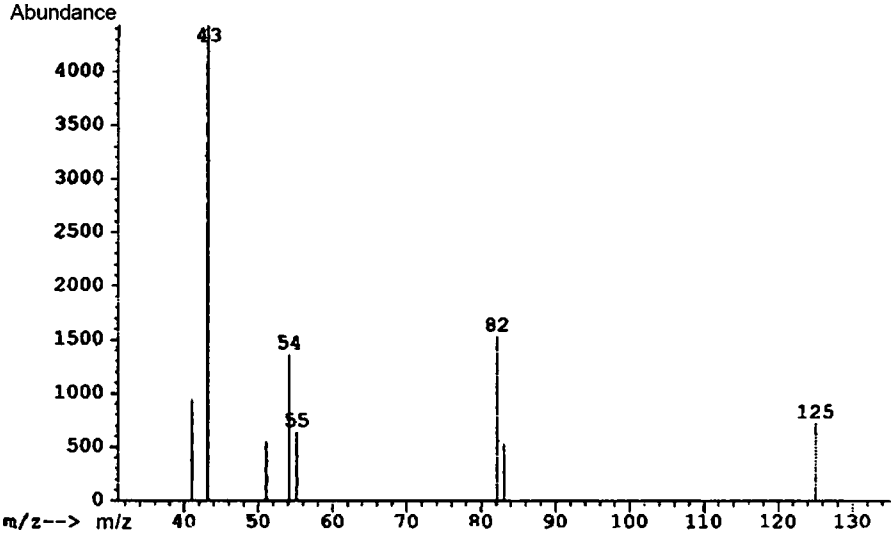


**Figure 5.** Mass spectrum of 2-acetyltetrahydropyridine.

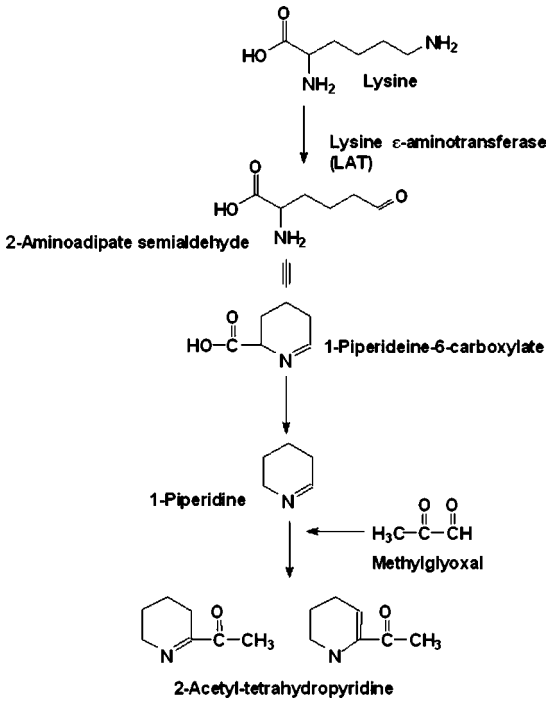


**Figure 6.** Proposed mechanism for 2-acetyltetrahydropyridine production by L-lysine-ϵ-aminotransferase from the *Streptomyces clavuligerus* gene (*lat*) expressed in *Escherichia coli*.

The deduced amino acid sequence of *S. clavuligerus* LAT showed extensive similarity to LAT from several bacteria such as *Amycolatopsis lactamdurans* (77% identity), *M. tuberculosis* (57% identity), *Mycobacterium avium* (55% identity), and *Desulfotalea psychrophila* (45% identity). However, it displayed less similarity (23% identity) to *Flavobacterium lutescens*. It also exhibited significant similarity (approximately 30% identity) to the eukaryotic aminotransferases, such as 4-aminobutyrate aminotransferase from *Saccharomyces cerevisiae*, *Danio rerio* (zebrafish), and *Homo sapiens* (human). Like other aminotransferases, the LAT is also a pyridoxal 5′-phosphate (PLP)-dependent enzyme. On the basis of amino acid similarity and 3D structures, PLP-dependent enzymes have been divided into four different families: α family, β family, D-alanine aminotransferase family, and alanine racemase family (*27*). *S. clavuligerus* LAT belongs to aminotransferase subfamily II of the α family. A search through the available protein motif databases using the Sequence Motif Search at GenomeNet webserver revealed that the sequence of the PLP attachment site is located between residues 272 and 309. As shown in **Figure 1**, the PLP attachment site of *S. clavuligerus* LAT exhibited high similarity to those of several bacterial LAT. Similarities were found not only with LAT but also with other aminotransferases, such as 4-aminobutyrate aminotransferase and acetylornithine aminotransferase. During catalysis, the PLP coenzyme is covalently linked to a lysine residue through a Schiff base linkage. On the basis of the sequence comparison, Lys 304 of *S. clavuligerus* LAT is likely to be involved directly in PLP binding.

**Purification and Characterization of the Recombinant LAT.** The coding region of the *lat* gene was cloned into the pET system under the control of the T7 promoter. The BL21 host cell containing the target insert gene was induced by IPTG. The recombinant LAT was overexpressed in *E. coli* BL21 (DE3) as inclusion bodies and then solubilized with 6 M urea solution. However, the refolding procedure of the recombinant enzyme did not allow the production of functional active LAT. Therefore, *lat* gene was inserted into pGEM-7Zf (+) and expressed in *E. coli* JM109. The recombinant LAT was purified from the cytoplasmic fraction of *E. coli* JM109 (pGEM-scLAT) by ammonium sulfate (30−60%) precipitation and Sephacryl S-200 column chromatography. After examination of the purified protein preparation by SDS−PAGE, a single band with a molecular mass of 49.8 kDa was observed (**Figure 2**). It is in good agreement with the value estimated from the deduced amino acid sequence of LAT. As shown in **Figure 3**, the purified recombinant LAT had the similar optimal pH (6.5−7.5) and optimal temperature (25 °C) as those of wild-type LAT (*28*). However, the recombinant LAT showed higher specific activity [1.78 units (mg of protein)$^{-1}$] than that of wild-type LAT [0.12 unit (mg of protein)$^{-1}$]. Similar to most aminotransferases, LAT from *S. clavuligerus* is derived from a single ORF and is active as a monomer (*25*). The purified recombinant LAT was used to transform lysine into P6C in this experiment.

**P6C Preparation.** The formation of P6C is dose-dependent on lysine concentration as shown in **Figure 4**. A 100 mL reaction mixture containing 5 mmol of L-lysine and purified LAT produced 1.7 mmol of P6C, which presented 34% conversion to P6C. Recently, Rius et al. (*29*) found that addition of lysine into *S. clavuligerus* culture could induce LAT and

subsequently the biosynthesis of P6C. Lysine was found to be transformed to 1-piperideine-6-carboxylic acid by the purified *S. clavuligerus* LAT expressed in *E. coli*.

**Identification of 2-Acetyltetrahydropyridine as Principal Odorant in the Reaction Mixture of P6C and Methylglyoxal.** In the model system of P6C and methylglyoxal, volatile compounds were extracted with methylene chloride and analyzed by GC-MS. A major peak with the mass spectrum having a base peak at *m*/*z* 43 followed by 82, 54, 55, 82, and 125 fragment ions was noted **(Figure 5**). The breakdown of the acetyl moiety $CH_3-CO$ leads to the formation of fragmentation ion at *m*/*z* 82. A further degradation of methylene moiety $CH_2{=}CH_2$ results in the formation of the *m*/*z* 54 fragment. This peak was therefore identified as 2-acetyltetrahydropyridine when its mass spectrum was compared with library data and that of Hofmann and Schieberle (*16*). In addition to 2-acetyltetrahydropyridine, several unidentified compounds were observed. By comparison with the external standard 2,4,6-trimethylpyridine, the amount of 2-acetyltetrahydropyridine in the model system of P6C and methylglyoxal was estimated to be in the range of 2.4−3.6 mmol. which accounted for 1.2−1.8% conversion rate of 1-piperideine-6-carboxylic acid to 2-acetyltetrahydropyridine.

Biosynthesis of 2-acetyltetrahydropyridine has been reported to be associated with lactic acid bacterium in wine (*30*). Since lysine was found to stimulate the formation of 2-acetyltetrahydropyridine, they proposed that acetylation at the C-2 position of the 1-piperideine intermediate by the accumulated acyl-CoA (or similar) derivatives may lead to the formation of the acylated mousy N-heterocycles. A hypothetical pathway leading from 1-pyrroline and hydroxy-2-propanone to 2-acetyltetrahydropyridine was proposed by Hofmann and Schieberle (*16*). Our data showed that P6C, derived from the conversion of L-lysine by the purified recombinant LAT, might react directly with methylglyoxal and lead to the formation of 2-acetyltetrahydropyridine. On the other hand, P6C may also degrade to 1-piperideine and condense with methylglyoxal to form 2-acetyltetrahydropyridine **(Figure 6**). To the best of our knowledge, the proposed P6C/methylglyoxal pathway represents the first report of a biological mechanism by which 2-acetyltetrahydropyridine may be synthesized in vitro by L-lysine-ϵ-aminotransferase.

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