Biosci. Biotechnol. Biochem., **71** (7), 1607–1615, 2007

Synthesis of 4-Hydroxyisoleucine by the Aldolase–Transaminase Coupling Reaction and Basic Characterization of the Aldolase from *Arthrobacter simplex* AKU 626

Jun Ogawa,[1] Hiroyuki Yamanaka,[1] Junichi Mano,[1] Yuko Doi,[1] Nobuyuki Horinouchi,[1] Tomohiro Kodera,[2] Noriki Nio,[2] Sergey V. Smirnov,[3] Natalya N. Samsonova,[3] Yury I. Kozlov,[3] and Sakayu Shimizu[1,†]

[1]*Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Kitashirakawa-oiwakecho, Sakyo-ku, Kyoto 606-8502, Japan*
[2]*Institute of Life Sciences, Ajinomoto Co., Inc., Suzuki-cho, Kawasaki-ku, Kawasaki-shi 210-8681, Japan*
[3]*Ajinomoto-Genetika Research Institute, 1st Dorozhny pr. 1, Moscow 113545, Russia*

Received November 21, 2006; Accepted March 29, 2007; Online Publication, July 7, 2007
[doi:10.1271/bbb.60655]

***Arthrobacter simplex* AKU 626 was found to synthesize 4-hydroxyisoleucine from acetaldehyde, α-ketobutyrate, and L-glutamate in the presence of *Escherichia coli* harboring the branched chain amino acid transaminase gene (*ilvE*) from *E. coli* K12 substrain MG-1655. By using resting cells of *A. simplex* AKU 626 and *E. coli* BL21(DE3)/pET-15b-*ilvE*, 3.2 mM 4-hydroxyisoleucine was produced from 250 mM acetaldehyde, 75 mM α-ketobutyrate, and 100 mM L-glutamate with a molar yield to α-ketobutyrate of 4.3% in 50 mM Tris–HCl buffer (pH 7.5) containing 2 mM $MnCl_2 \cdot 4H_2O$ at 28 °C for 2 h. An aldolase that catalyzes the aldol condensation of acetaldehyde and α-ketobutyrate was purified from *A. simplex* AKU 626. Mn^{2+} and pyridoxal 5′-monophosphate were effective in stabilizing the enzyme. The native and subunit molecular masses of the purified aldolase were about 180 and 32 kDa respectively. The N-terminal amino acid sequence of the purified enzyme showed no significant homology to known aldolases.**

Key words: 4-hydroxyisoleucine; aldolase; transaminase; *Arthrobacter simplex*; antidiabetics

In 1990, Sharma *et al.* reported for the first time that fenugreek seeds lowered serum lipids. Through that research, fenugreek seeds were found to contain 4-hydroxyisoleucine (HIL) as a unique major free amino acid; it has been characterized as one of the active ingredients of these seeds.[1–5] In 2000, Broca *et al.* discovered that the major HIL isomer in fenugreek seeds, 2*S*,3*R*,4*S*-HIL, induces glucose-induced insulin secretion through a direct effect on pancreatic β cells in rats and humans.[6] Furthermore, in a new rat model of type II diabetes, 2*S*,3*R*,4*S*-HIL partly corrected hyperglycemia and glucose tolerance.[6] Due to this insulinotropic activity, this isomer may be considered a novel orally-active drug with potential for the treatment of insulin-independent diabetes mellitus. Since the extraction of HIL from fenugreek seeds is done at low yield (about 150 mg HIL from 1 kg of seeds[1]), the pharmaceutical industry requires a more efficient method for the synthesis of HIL.

We attempted to synthesize HIL by the aldolase–transaminase coupling reaction. The coupling reaction consists of two reactions. In the first reaction, an aldolase catalyzed the aldol condensation of acetaldehyde and α-ketobutyrate. In the second reaction, a transaminase catalyzed amination of the aldol condensation product, 4-hydroxy-3-methyl-2-keto-pentanoic acid, to HIL (Fig. 1). Aldolase is the key enzyme, since it can generate two of three chiral centers in HIL. The aldolase catalyzing the first reaction, 4-hydroxy-2-keto-pentanoic acid aldolase (HKP aldolase), has been found in cell-free extract of *Escherichia coli* W3110.[7] The enzyme, however, catalyzes the condensation of acetaldehyde and α-ketobutyrate only under alkaline reaction conditions, in which the condensation product, 4-hydroxy-3-methyl-2-keto-pentanoic acid, is easily dehydrated to undesirable products (our unpublished results). HKP aldolase is involved in the later stages of many bacterial catabolic pathways responsible for the degradation of aromatic compounds *via meta*-cleavage pathways, and is classified as a class I aldolase.[8–12] Hence we screened for an aldolase-producing microorganism suitable for the aldolase–transaminase coupling reaction among aromatic compound-assimilating microorganisms and in the strains of our culture collection in the presence of *Escherichia coli* harboring the branched chain amino

† To whom correspondence should be addressed. Fax: +81-75-753-6128; E-mail: sim@kais.kyoto-u.ac.jp

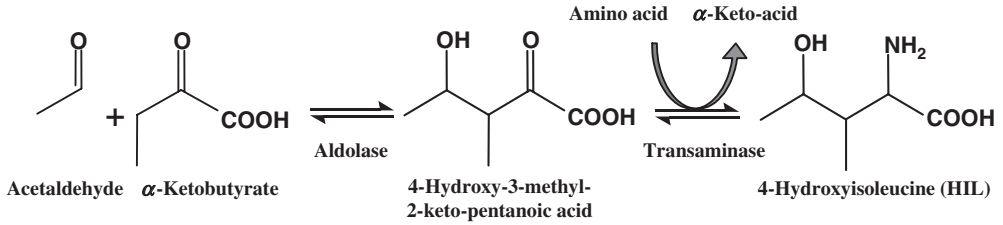

Fig. 1. Synthetic Strategy for HIL by the Aldolase–Transaminase Coupling Reaction.

acid transaminase gene (*ilvE*) from *E. coli* K12 substrain MG1655.

In this paper, we describe the screening of an aldolase-producing bacterium that is suitable for the aldolase–transaminase coupling reaction and the optimization of HIL synthesis using resting cells of *Arthrobacter simplex* AKU 626 and *E. coli* BL21(DE3)/pET-15b-*ilvE*, as well as the basic characterization of the aldolase purified from *A. simplex* AKU 626.

Materials and Methods

Chemicals. An authentic sample of HIL was obtained from the Institute of Life Sciences., Ajinomoto Co., Inc. (Kawasaki, Japan). 3-(2-Hydroxyphenyl)propionic acid and 3-(4-methylphenyl)propionic acid were purchased from Lancaster Industrial Chemical Manufacturers (Morecambe, England). (*S*)-2-Aminobutyric acid, catechol, α-ketobutyrate, acetaldehyde, and L-glutamate were purchased from Wako Pure Chemical Industries (Osaka, Japan.). All other chemicals used were of analytical grade and are commercially available.

Microorganisms and culture conditions. All media described below were adjusted to pH 7.0 unless otherwise stated. Microorganisms assimilating aromatic compounds were isolated by enrichment culture. The medium (medium A) used was composed of 0.1% (w/v) KH_2PO_4, 0.1% (w/v) K_2HPO_4, 0.03% (w/v) $MgSO_4 \cdot 7H_2O$, 0.01% (w/v) yeast extract, 0.2% (w/v) NH_4Cl, and 0.15% (w/v) carbon source, and was incubated at 28 °C. The same medium was used for the cultivation of aromatic compound-assimilating microorganisms. Catechol, 3-(2-hydroxyphenyl)propionic acid, or 3-(4-methylphenyl)propionic acid was used as a carbon source. Microorganisms obtained from the culture collection of the Faculty of Agriculture of Kyoto University (AKU) were also examined. The medium for bacteria and yeasts (medium B) comprised 1.5% (w/v) peptone, 0.1% (w/v) yeast extract, 0.3% (w/v) K_2HPO_4, 1.0% (w/v) glucose, 0.2% (w/v) NaCl, and 0.01% (w/v) $MgSO_4 \cdot 7H_2O$. The medium for actinomycetes comprised 0.4% (w/v) soluble starch, 0.4% (w/v) yeast extract, and 1.0% (w/v) malt extract. The medium for molds and basidiomycetes comprised 0.5% (w/v) peptone, 0.3% (w/v) yeast extract, 1.0% (w/v) glucose, and 0.3% (w/v) malt extract (pH 6.0). *E. coli* BL21(DE3)/pET-15b-*ilvE*, harboring the branched chain amino acid transaminase gene from *E. coli* K-12 substrain MG1655, was obtained from the Institute of Life Sciences, Ajinomoto Co., Inc., and cultivated in Luria Bertrani (LB) medium containing 1.0% (w/v) tryptone, 0.5% (w/v) yeast extract, and 1.0% (w/v) NaCl in tap water (pH 7.2).

Screening methods. E. coli BL21(DE3)/pET-15b-*ilvE* was cultivated at 37 °C with shaking (120 strokes/min) in a 2-l shaking flask containing 500 ml of LB medium until OD_{555} reached 1.0. Then the transaminase gene was induced by the addition of isopropyl β-D-thiogalactoside to the medium at a final concentration of 1 mM, and the culture was subsequently incubated at 37 °C. After 4 h, the cells were harvested by centrifugation (12,000 × *g* for 20 min at 4 °C, High Mac CR21; Hitachi, Tokyo), washed twice with physiological saline, centrifuged again, and re-suspended in 16 ml of physiological saline. The microorganisms isolated or obtained from the type culture collection were inoculated into 5 ml of each medium described above, and then cultivated with shaking (300 strokes/min) at 28 °C for 1–2 d for screening of aldolase activity. After cultivation, the culture broths (5 ml each) were added to the *E. coli* BL21(DE3)/pET-15b-*ilv* cell suspension (200 µl) described above. The combined cell suspension obtained was centrifuged (2,150 × *g* for 10 min at room temperature, LC-100; Tomy Seiko, Tokyo), and the cells were resuspended in physiological saline and centrifuged again. Resting cells consisting of the various microorganisms for aldolase screening and *E. coli* BL21(DE3)/pET-15b-*ilvE* were examined as biocatalysts for the synthesis of HIL. The aldolase–transaminase coupling reaction was started by the addition of a reaction mixture (0.5 ml), comprising a 50 mM potassium phosphate buffer (pH 7.0), 1% (w/v) acetaldehyde (227 mM), 1% (w/v) α-ketobutyrate (68 mM), and 1% (w/v) L-glutamate (68 mM) to the resting cells in a test tube (12 × 100 mm). The reaction was carried out at 28 °C with shaking (300 strokes/min) for 12–24 h. The acetaldehyde and α-ketobutyrate solutions were acidic and were neutralized with 2 N NaOH prior to use. After incubation, the reaction mixture was centrifuged (2,150 × *g* for 10 min at room temperature, LC-100), and the supernatant was diluted 1:500 with MilliQ-filtered de-ionized water, and 20 µl of the diluted sol-

ution obtained was reacted with AccQ-Tag fluorescence derivatization reagent (Waters, Milford, MA) to derivatize HIL to AccQ-Tag-derivated HIL, and 10 µl of the reaction mixture was subjected to amino acid analysis.

Analytical methods. Quantitative analysis of AccQ-Tag-derivatized HIL was performed with an amino acid analyzer (Waters 2695 Separation Module System; Waters) equipped with an AccQ-Tag column (3.9 × 150 mm; Waters). The AccQ-Tag-derivatized HIL was eluted with AccQ-Tag Eluent A (Waters) and acetonitrile at a flow rate of 1.0 ml/min. The gradient program used was as follows: initial 0% acetonitrile (100% AccQ-Tag Eluent A); linear gradient for 0.5 min to 1% acetonitrile; linear gradient for 17.5 min to 5% acetonitrile; linear gradient for 1 min to 9% acetonitrile; linear gradient for 10.5 min to 17% acetonitrile; linear gradient for 3.5 min to 60% acetonitrile; hold for 3 min at 60% acetonitrile; linear gradient for 6 s to 0% acetonitrile, and equilibration for 15 min at 0% acetonitrile. The eluted compound was monitored with a fluorescence detector at 295 nm with the emission induced by excitation at 250 nm.

Optimization of the reaction conditions for HIL synthesis using resting cells of A. simplex *AKU 626. A. simplex* AKU 626 was cultivated at 28 °C with shaking (120 strokes/min) in a 2-l shaking flask containing 500 ml of medium B for 36–48 h. Five ml of the culture broth was sampled and used for the reaction. The reaction was carried out under the conditions described under "Screening methods." If necessary, the reaction was terminated by the addition of 50 µl of 15% (v/v) perchloric acid, and the solution was neutralized with 450 µl of 500 mM potassium phosphate buffer (pH 7.0). The effects of cultivation time, reaction time, reaction pH, cofactors, and amino donors on HIL synthesis were examined under the same conditions, except for the condition tested.

Stereoisomer analysis of HIL by liquid chromatograph mass spectrometer (LC–MS). Enzymatically prepared HIL was reacted with AccQ-Tag fluorescence derivatization reagent (Waters), and AccQ-Tag-derivated HIL was separated using a high pressure liquid chromatograph, Alliance 2695 (Waters) equipped with column LUNA C18 (150 × 2 mm, 3 µm; Phenomenex, Torrance, CA) at 26 °C. The mobile phase contained 10 mM CH_3COONH_4 at pH 6.8. The flow rate was 0.3 ml/min. Detection was performed by positive mode electrospray ionization (ESI) in a Quattro-Micro tandem quadrupole mass-spectrometer (Waters). The source conditions were: capillary voltage, 2.0 kV; cone voltage, 20 V; source temperature, 119 °C; dessolvation temperature, 450 °C; cone gas flow, 60 l/h; dessolvation gas flow, 659 l/h.

Purification of the aldolase from A. simplex *AKU 626.* All procedures were carried out at 0–5 °C, and a 20 mM Tris–HCl buffer (pH 7.4) containing 0.1 mM dithiothreitol, 1 mM $MnCl_2 \cdot 4H_2O$, 0.05 mM pyridoxal 5′-phosphate (PLP), and 5% ethylene glycol (buffer A) was used as the standard buffer.

Step 1: Preparation of a cell-free extract. *A. simplex* AKU 626 was cultivated in 10 liters of Medium B at 28 °C for 48 h with shaking, and the cells were collected by centrifugation at 12,000 × *g* for 20 min at 4 °C (High Mac CR21). The cells obtained (about 100 g wet-weight) were suspended in 150 ml of buffer A, and then ultrasonically disrupted for 10 min (19 kHz, Insonator model 201M; Kubota, Osaka, Japan) with cooling by water and ice. After centrifugation at 12,000 × *g* for 20 min at 4 °C (High Mac CR21), the supernatant was used as a cell-free extract.

Step 2: Ammonium sulfate fractionation. The cell-free extract was fractionated with solid ammonium sulfate. The precipitate obtained at 70–80% ammonium sulfate saturation was collected by centrifugation at 16,000 × *g* for 20 min (High Mac CR21) and then dissolved buffer A (15 ml). The enzyme solution (16 ml) was dialyzed against 8 liters of buffer A for 12 h.

Step 3: Mono Q column chromatography. The dialyzed enzyme solution was applied to a Mono Q HR 10/10 column (Amersham Pharmacia Biotech, Uppsala, Sweden) equilibrated with buffer A. After it was washed with buffer A, the enzyme was eluted with a linear gradient of 1–0 M NaCl in 120 ml of buffer A. The active fractions were combined (5.5 ml).

Step 4: Phenyl-Superose column chromatography. After the addition of ammonium sulfate to 50% saturation, the enzyme solution was applied to a phenyl-Superose column, HR 5/5 (Amersham Pharmacia Biotech), equilibrated with buffer A containing ammonium sulfate (50% saturation) (buffer B). After the column was washed with buffer B, the enzyme was eluted with a linear gradient of ammonium sulfate, 50–0% saturation, in 30 ml of buffer A. The active fractions were combined (1.5 ml).

Step 5: Alkyl-Superose column chromatography. After the addition of ammonium sulfate to 50% saturation, the enzyme solution was applied to an alkyl-Superose column, HR 5/5 (Amersham Pharmacia Biotech), equilibrated with buffer B. After it was washed with buffer B, the enzyme was eluted with a linear gradient of ammonium sulfate, 50–0% saturation, in 30 ml of buffer A. The fractions containing the enzyme activity (500 µl) were concentrated to 50 µl by ultrafiltration with a Centricon 10 (Amicon, Beverly, MA).

Step 6: Superdex 200 column chromatography. The enzyme solution was applied to a Superdex 200 HR 10/30 column (Amersham Pharmacia Biotech) equilibrated with buffer A containing 200 mM NaCl (buffer C). The enzyme was eluted with 40 ml of buffer C. The fractions (0.5 ml) containing the enzyme activity were used for further characterization.

Table 1. Microorganisms Producing More Than 1 mM HIL (a) and Showing High Productivity with Good Reproducibility (b)

(a)

	Bacteria	Yeasts	Actinomycetes	Molds	Basidiomycetes	Soil isolates
Screened	176	248	78	177	57	840
Showing HIL production (> 1 mM)	12	18	0	0	1	10

(b)

AKU number	Strain	Origin	Class	HIL concentration (μM)
105	*Alcaligenes denitrificans* subsp. *denitrificans*		Bacterium	1580
626	*Arthrobacter simplex*	IFO12069	Bacterium	2240
995	*Ochrobactrum anthropi* A37a		Bacterium	1470
4303	*Hansenula anomala*	IFO 0149	Yeast	1040

Enzyme assays. Because it was impossible to measure the amount of the aldolase condensation product, 4-hydroxy-3-methyl-2-keto-pentanoic acid, it was difficult directly to evaluate the aldolase activity. Hence the apparent aldolase activity was evaluated by the production of HIL through the aldolase–transaminase coupling reaction. The standard conditions for HIL-producing activity assay were as follows: The reaction mixture, 200 μl, comprised 50 μl of enzyme solution, 30 μl of *E. coli* BL21(DE3)/pET-15b-*ilvE* cell suspension as described above, 50 mM Tris–HCl buffer (pH 7.4), 0.2 mM PLP, 250 mM acetaldehyde, 75 mM α-ketobutyrate, and 100 mM L-glutamate. Each reaction mixture was incubated at 28 °C for 12 h. Under the conditions employed, the rate of 4-hydroxy-3-methyl-2-keto-pentanoic acid amination was assumed to be proportionate to the rate of 4-hydroxy-3-methyl-2-keto-pentanoic acid synthesis. Thus the specific activity was indirectly estimated by the rate of HIL formation. After incubation, the reaction mixture was centrifuged at 14,000 × *g* for 10 min at 4 °C (MX-150; Tomy Seiko, Tokyo), and then the supernatant was analyzed as described above. One unit of aldolase activity corresponded to the formation of 1 μmol of HIL/min.

Analytical methods. Protein concentration was determined with a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was performed in a 12.5% polyacrylamide gel using the Tris–glycine buffer system. The native and subunit molecular masses of the enzyme were determined by comparison of the mobility of the purified enzyme on gel filtration chromatography on Superdex 200 HR 10/30 and SDS–PAGE respectively with those of the standard protein (MW-Marker, HPLC, for gel filtration; Oriental Yeast, Osaka, Japan, and M. W. marker III for SDS–PAGE; Daiichi Kagaku Yakuhin, Tokyo). The N-terminal amino acid sequence of the purified enzyme was analyzed by automated Edman degradation with a 491HT protein sequencer (Applied Biosystems, Foster City, CA).

Results and Discussion

Screening of the aldolase-producing microorganisms useful for synthesis of HIL by the aldolase–transaminase coupling reaction

The aromatic compound-assimilating microorganisms isolated from soils (840 strains), and the stocked cultures, including bacteria (176 strains), yeasts (248 strains), actinomycetes (78 strains), molds (177 strains), and basidiomycetes (57 strains), were examined as biocatalysts for the synthesis of HIL by the aldolase–transaminase coupling reaction. The isolated microorganisms (10 strains) and the type-cultures (31 strains: bacteria 12, yeasts 18, and basidomycete 1) were found to synthesize HIL at more than 1 mM. These 41 strains were re-examined, and reproducibility for synthesis of HIL was observed for only four strains (Table 1). The highest HIL synthesis was observed for *Arthrobacter simplex* AKU 626. From 1% (w/v) acetaldehyde (227 mM), 1% (w/v) α-ketobutyrate (68 mM), and 1% (w/v) L-glutamate (68 mM), 2.2 mM HIL was synthesized in 12 h in the presence of resting cells of *A. simplex* AKU 626 and *E. coli* BL21(DE3)/pET-15b-*ilvE* under the screening conditions. *A. simplex* AKU 626 was therefore selected and used for further investigation.

Effect of the cultivation time of A. simplex *AKU 626 on the aldolase–transaminase coupling reaction*

Culture broth (5 ml) was taken during the cultivation of *A. simplex* AKU 626 at 6-h intervals, and was used to examine the effect of cultivation time on the aldolase–transaminase reaction. High levels of HIL synthesis were observed when it was cultivated for 24–48 h, which corresponded to the mid-log and stationary growth phases. The addition of aromatic compounds such as catechol, 3-(2-hydroxyphenyl)propionic acid, and 3-(4-methylphenyl)propionic acid did not increase the amount of HIL synthesized, indicating that the enzyme(s) involved in the aldol condensation of acetaldehyde and α-ketobutyrate are not induced by these compounds.

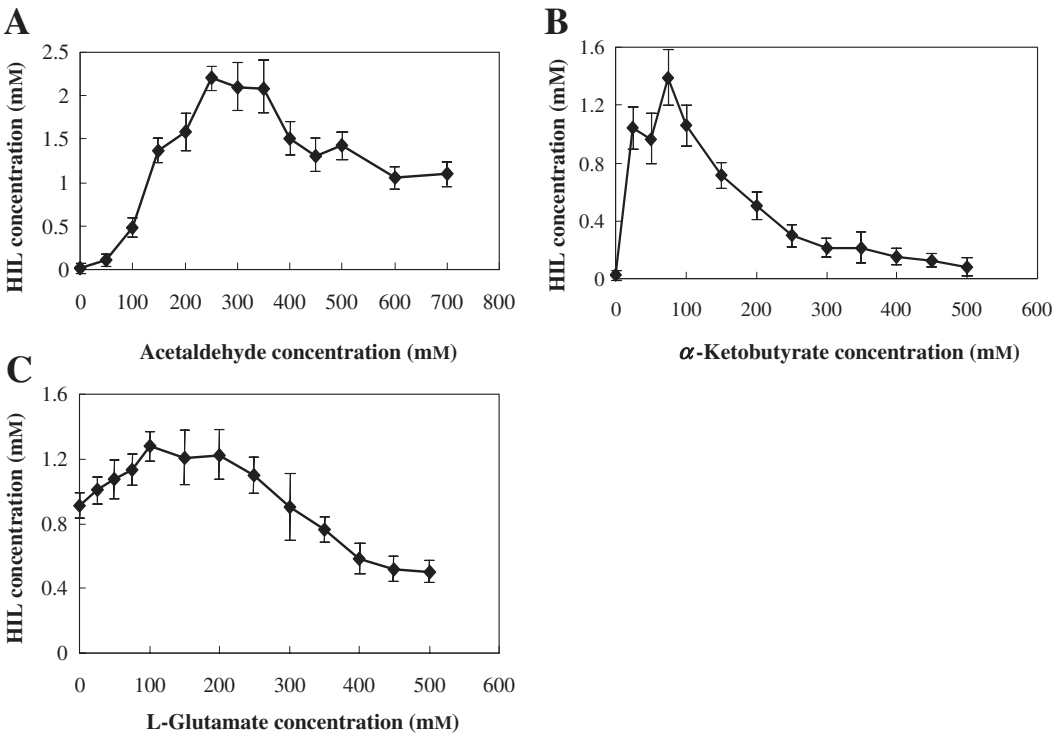

Fig. 2. Effects of Substrate Concentrations on HIL Synthesis.

A, acetaldehyde; B, α-ketobutyrate; C, L-glutamate. Reactions were carried out under standard conditions in a reaction mixture (0.5 ml) comprising a 50 mM potassium phosphate buffer (pH 7.0), 1% (w/v) acetaldehyde (227 mM), 1% (w/v) α-ketobutyrate (68 mM), and 1% (w/v) L-glutamate (68 mM), with the following exceptions: B, 250 mM acetaldehyde was used; C, 250 mM acetaldehyde and 75 mM α-ketobutyrate were used. Each point represents the mean ± S.E. (standard error of the mean) for triplicate samples in duplicate experiments.

Optimization of the reaction conditions for HIL synthesis

Optimum concentrations of the substrates: The optimum concentrations of acetaldehyde, α-ketobutyrate, and L-glutamate were found to be 250, 75, and 100 mM, respectively (Fig. 2). HIL was also synthesized without the addition of L-glutamate (Fig. 2C), indicating that an endogenous amino donor probably exists in the resting cells.

Effect of pH: The highest HIL synthesis was observed in a 50 mM Tris–HCl buffer, pH 7.5 (Fig. 3). A lower HIL synthesis was observed at the same pH in a 50 mM potassium phosphate buffer. HIL synthesis decreased with increasing concentrations of a potassium phosphate buffer.

Effects of metal ions: The effects of various metal ions (2 mM) on HIL synthesis were examined. HIL synthesis was enhanced by the addition of Co^{2+}, Mn^{2+}, Mg^{2+}, or Fe^{2+}, and the relative yields were 122, 140, 118, and 117% respectively. The other metal ions tested (Na^{+}, K^{+}, Ca^{2+}, Zn^{2+}, and Cu^{2+}) were not effective on HIL synthesis.

Effects of amino donors: Various amino donors were examined in place of L-glutamate. Among the compounds listed in Table 2, high levels of HIL synthesis were achieved with aliphatic and aromatic amino acids, such as valine, leucine, norvaline, phenylalanine, and phenylglycine. (*S*)-2-Aminobutyric acid, a by-product of the aldolase–transaminase coupling reaction (Fig. 6), also served as an amino donor in this reaction (data not shown).

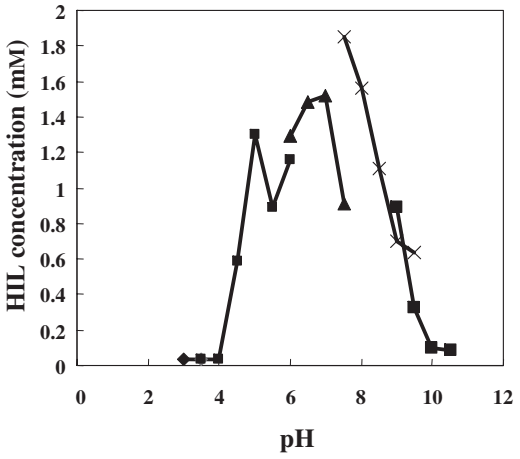

Fig. 3. Effect of Reaction pH on HIL Synthesis.

Reactions were carried out under standard conditions, except for the buffers (50 mM) used: AcONa–HCl (pH 3–3.5); AcOH–AcONa (pH 3.5–6.0); KPB (pH 6.0–7.5); Tris–HCL (pH 7.5–9.5), and Borate (pH 9.0–10.5). Each reaction mixture was incubated at 28 °C for 12 h.

Table 2. Effects of Amino Donors on HIL Synthesis from *A. simplex* AKU 626

Amino donor	Relative activity (%)	Amino donor	Relative activity (%)	Amino donor	Relative activity (%)
L-Glycine	28	L-Methionine	109	DL-Norvaline	150
L-Alanine	94	L-Cysteine	5	L-Kynurenine	6
DL-Valine	128	L-Glutamate	100	L-Ornithine	6
L-Leucine	154	L-Glutamine	94	L-Histidinol	6
L-Isoleucine	109	L-Aspartate	94	L-2-Aminoadipic acid	9
L-Serine	100	L-Asparagine	26	Taurine	11
L-Threonine	75	L-Lysine	43	L-2-Phenylglycine	123
L-Tyrosine	64	L-Histidine	25	4-Aminobutyric acid	56
L-Phenylalanine	155	L-Arginine	12	Ammonium sulfate	42
L-Tryptophan	106	β-Alanine	75	None	12

Activity with L-glutamate was defined as 100%.

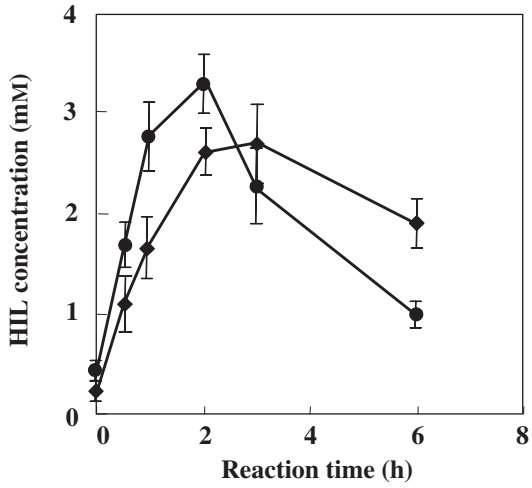

Fig. 4. Time Course of HIL Synthesis by the Aldolase–Transaminase Coupling Reaction with Resting Cells of *A. simplex* AKU 626.

The reaction was carried out in 50 mM Tris–HCl buffer (pH 7.5) containing 250 mM acetaldehyde, 75 mM α-ketobutyrate, 100 mM L-glutamate, and 2 mM $MnCl_2 \cdot 4H_2O$ at 28 °C. Each point represents the mean ± S.E. for triplicate samples in duplicate experiments. Closed circles, reaction with resting cells of *A. simplex* AKU 626 and *E. coli* BL21(DE3)/pET-15b-*ilvE*. Closed square, reaction with resting cells of *A. simplex* AKU 626.

Time course of HIL synthesis by the aldolase–transaminase coupling reaction with resting cells of A. simplex *AKU 626*

The time course of HIL synthesis by resting cells of *A. simplex* AKU 626 and *E. coli* BL21(DE3)/pET-15b-*ilvE* was investigated under optimum reaction conditions, with 250 mM acetaldehyde, 75 mM α-ketobutyrate, and 100 mM L-glutamate in 50 mM Tris–HCl buffer (pH 7.5) containing 2 mM $MnCl_2 \cdot 4H_2O$ at 28 °C. HIL synthesis was maximum (3.2 mM) at 2 h, and decreased with longer reaction times (Fig. 4). The amount of HIL produced corresponding to 470 mg in 1-L of the reaction mixture is comparable to the amount of extracted HIL from 3 kg fenugreek seeds (about 150 mg from 1 kg of fenugreek seeds). Even in the absence of transaminase-expressing *E. coli* BL21(DE3)/pET-15b-*ilvE* cells, HIL was synthesized, indicating that an endogenous transaminase exists in *A. simplex* cells.

Basic characterization of the aldolase from A. simplex *AKU 626*

The aldolase activity of the cell-free extract of *A. simplex* AKU 626 significantly decreased during dialysis. Because there have been reports that aldolases require divalent metal ions, the effects of metal ions were investigated. Mn^{2+}, Co^{2+}, and Cd^{2+} were found to be effective for maintaining aldolase activity in cell-free extract. Among these, Mn^{2+} was selected for further experiments because it did not produce any color or precipitate which would disturb purification by column chromatography or protein analysis on measuring absorbance. The effects of various cofactors and organic acids were also investigated. Aldolase activity was maintained by the addition of pyridoxal 5′-monophosphate (PLP) to the dialysis buffer. The other cofactors (NADH, NADPH, FAD, and thiamine pyrophosphate) and the organic acids (ascorbate, succinate, and citrate) did not have significant effects. To maintain aldolase activity, PLP (0.05 mM) and Mn^{2+} (1 mM) were added to the buffer during purification.

The aldolase was purified to homogeneity through five successive steps from cell-free extract of *A. simplex* AKU 626. A typical purification is shown in Table 3. The final preparation gave a single band on SDS–PAGE (Fig. 5), and showed specific activity of 0.045 U/mg. The native protein showed a molecular mass of 180 kDa by gel filtration and of 32 kDa by SDS–PAGE analysis, indicating that the aldolase was a hexameric protein.

The N-terminal amino acid sequence of the enzyme was determined to be as follows: PFPVELPDNFAKR-VTDSXSAQV. This sequence showed no significant homology to known aldolases on a BLAST database search. This suggests that the aldolase catalyzing HIL production was not HKP aldolase but a novel aldolase.

The optimum reaction pH and temperature for the purified enzyme in the presence of PLP and Mn^{2+} were pH 7.0 (in a 50 mM potassium phosphate buffer) and 25 °C. More than 80% of the initial activity of the enzyme was retained in a pH range of 6.0–8.0 after incubation at 28 °C for 60 min. The enzyme was stable below 30 °C after incubation at pH 8.0 for 60 min, but only 20% of the original activity was retained at 35 °C after incubation at pH 8.0 for 60 min.

Table 3. Purification of the Aldolase

	Total protein (mg)	Total activity ($\times 10^{-3}$ U)	Specific activity ($\times 10^{-3}$ U/mg)	Yield (%)
Cell-free extract	5175	2453	0.47	100
Ammonium surfate fractionation	589	1518	2.58	62
Mono Q HR 10/10	39.4	63.3	1.61	2.6
Phenyl-Superose HR 5/5	1.74	21.2	12.2	0.87
Alkyl-Superose HR 5/5	0.054	7.95	148	0.32
Superdex 200 HR 10/30	0.033	1.51	44.8	0.06

Enzyme activity was evaluated by HIL production though aldolase–transaminase coupling reaction, as described in "Materials and Methods."

Fig. 5. SDS–PAGE Analysis of Purified Aldolase from *A. simplex* AKU 626.

Lane A, the purified enzyme (approximately 0.5 μg) after Superdex 200 column chromatography. Lane B, molecular mass standards (approximately 20 μg); from top: phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa), aldolase (42 kDa), carbonic anhydrase (30 kDa), trypsine inhibitor (20 kDa), and lysozyme (14 kDa).

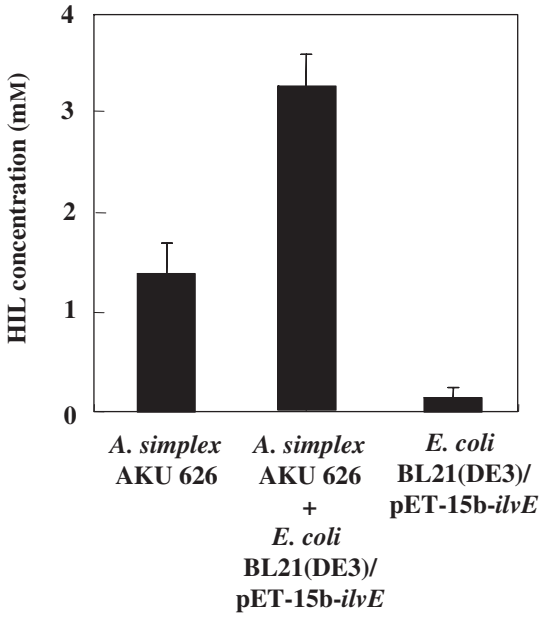

Fig. 6. HIL Synthesis by the Resting Cells of *A. simplex* AKU 626, *E. coli* BL21(DE3)/pET-15b-*ilvE*, and *A. simplex* AKU 626 and *E. coli* BL21(DE3)/pET-15b-*ilvE*.

HIL synthesis was carried out under optimum reaction conditions with 250 mM acetaldehyde, 75 mM α-ketobutyrate, 100 mM L-glutamate, and 2 mM $MnCl_2 \cdot 4H_2O$ at 28 °C for 1 h with resting cells of *A. simplex* AKU 626, *E. coli* BL21(DE3)/pET-15b-*ilvE*, or *A. simplex* AKU 626 and *E. coli* BL21(DE3)/pET-15b-*ilvE*. Each point represents the mean ± S.E. for triplicate samples in duplicate experiments.

We succeeded in synthesizing HIL by the aldolase–transaminase coupling reaction, and found that the aromatic compound-assimilating microorganisms isolated from soils and certain strains in our culture collection were useful for this reaction. This is the first report of HIL synthesis from acetaldehyde, α-ketobutyrate, and L-glutamate. After screening, *A. simplex* AKU 626 was selected as the most suitable aldolase-producing strain for HIL synthesis. By using this strain in the aldolase–transaminase coupling reaction in the presence of *E. coli* BL21(DE3)/pET-15b-*ilvE* harboring the branched chain amino acid transaminase gene from *E. coli* K-12 substrain MG1655, 3.2 mM HIL was synthesized under the optimum reaction conditions (Fig. 4). As shown in Fig. 6, even *A. simplex* AKU 626 or *E. coli* BL21(DE3)/pET-15b-*ilvE* alone produced HIL, indicating that *A. simplex* AKU 626 and *E. coli* BL21(DE3)/pET-15b-*ilvE* produce their own transaminase and aldolase respectively. However, the combination of these two strains resulted in much higher HIL production. Low transaminase activity in the resting cells of *A. simplex* AKU 626 might be a bottleneck in HIL production by the aldolase–transaminase coupling reaction, and this was ameliorated by the addition of resting cells of transaminase-expressing *E. coli* BL21(DE3)/pET-15b-*ilvE*.

HIL has three chiral centers, which generate eight stereoisomers. The stereostructure of the α-carbon of the HIL synthesized by the aldolase–transaminase coupling reaction might be of the L-form due to the stereospecificity of the transaminase. Therefore, there are four possible stereoisomers of HIL synthesized by the re-

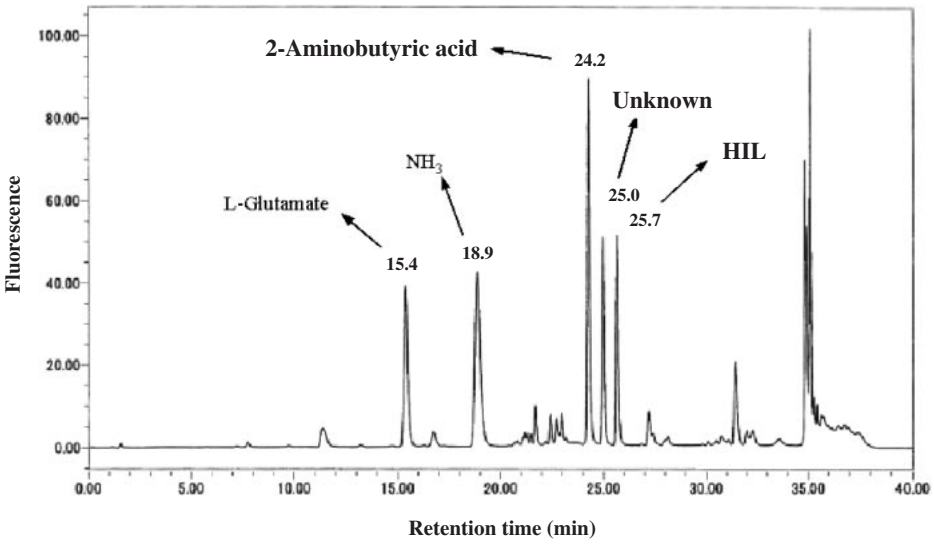

Fig. 7. Amino Acid Analysis Chromatogram of the Reaction Mixture of the Aldolase–Transaminase Coupling Reaction with Resting Cells of *A. simplex* AKU 626.

action. The stereostructure of HIL synthesized by the aldolase–transaminase coupling reaction was analyzed by LC–MS after it was reacted with AccQ-Tag fluorescence derivatization reagent. The HIL synthesized consisted of 2*S*,3*R*,4*S*-HIL and 2*S*,3*R*,4*R*-HIL in the ratio of 6:1. Only two isomers were found, and the major isomer was the same as the major isomer contained in fenugreek seeds.

Three peaks of the reaction products were observed in the chromatogram when the reaction mixture of the aldolase–transaminase coupling reaction with resting cells of *A. simplex* AKU 626 and *E. coli* BL21(DE3)/pET-15b-*ilvE* was analyzed with an amino acid analyzer. Two of these were identified as (*S*)-2-aminobutyric acid and HIL, and their retention times were 24.2 and 25.7 min respectively (Fig. 7). (*S*)-2-Aminobutyric acid was a by-product synthesized by the amination of α-ketobutyrate catalyzed by the transaminase. An unknown peak was observed at a retention time of 25.0 min. It was probably derived from a compound synthesized from acetaldehyde, α-ketobutyrate, and/or L-glutamate. Further identification of this unknown product is necessary to elucidate the reaction mechanism in detail.

Aldolases are divided into two classes: class I aldolases form a Schiff-base intermediate in the active site with the donor substrate, and class II aldolases use divalent metal ions as cofactors. The aldolase produced by *A. simplex* AKU626 was inhibited by a potassium phosphate buffer, and enhanced by Co^{2+}, Mn^{2+}, Mg^{2+}, and Fe^{2+}. Furthermore, the aldolase was stabilized by Mn^{2+}, Co^{2+}, or Cd^{2+} during purification. These results suggest that it is a class II aldolase. The *Arthrobacter* aldolase also required PLP to maintain its activity during purification. There have been some reports on PLP-dependent aldolases.[13–16] It is not clear that the *Arthrobacter* aldolase requires PLP as a coenzyme, because the transaminase in the reaction mixture also requires PLP as a cofactor. Further characterization, cloning, and expression of the aldolase are in progress.

References

1) Narender, T., Puri, A., Shweta, Khaliq, T., Saxena, R., Bhatia, G., and Chandra, R., 4-Hydroxyisoleucine an unusual amino acid as antidyslipidemic and antihyperglycemic agent. *Bioorg. Med. Chem. Lett.*, **16**, 293–296 (2006).
2) Handa, T., Yamaguchi, K., Sono, Y., and Yazawa, K., Effects of fenugreek seed extract in obese mice fed a high-fat diet. *Biosci. Biotechnol. Biochem.*, **69**, 1186–1188 (2005).
3) Flammang, A. M., Cifone, M. A., Erexson, G. L., and Stankowski, L. F. Jr., Genotoxicity testing of a fenugreek extract. *Food Chem. Toxicol.*, **42**, 1769–1775 (2004).
4) Fowden, L., Pratt, H. M., and Smith, A., 4-Hydroxyisoleucine from seed of *Torigonella foenum-graecum*. *Phytochemistry*, **12**, 1707–1711 (1973).
5) Haefele, C., Bonfils, C., and Sauvaire, Y., Characterization of a dioxgenase from *Torigonella foenum-graecum* involved in 4-hydroxyisoleucine biosynthesis. *Phytochemistry*, **44**, 563–566 (1997).
6) Broca, C., Manteghetti, M., Gross, R., Baissac, Y., Jacob, M., Petit, P., Sauvaire, Y., and Ribes, G., 4-Hydroxyisoleucine: effects of synthetic and natural analogues on insulin secretion. *Eur. J. Pharmacol.*, **390**, 339–345 (2000).
7) Pollard, J. R., Rialland, D., and Bugg, T. D. H., Substrate selectivity and biochemical properties of 4-hydroxy-2-keto-pentanoic acid aldolase from *Escherichia coli*. *Appl. Environ. Microbiol.*, **64**, 4093–4094 (1998).
8) Arai, H., Yamamoto, T., Ohnishi, T., Shimizu, T., Nakata, T., and Kudo, T., Genetic organization and characteristics of the 3-(3-hydroxyphenyl)propionic acid degradation pathway of *Comamonas testosteroni* TA441. *Microbiology*, **145**, 2813–2820 (1999).
9) Burlingame, R., and Chapman, P. J., Catabolism of

phenylpropionic acid and its hydroxy derivative by *Escherichia coli*. *J. Bacteriol.*, **155**, 113–121 (1983).
10) Burlingame, R., and Chapman, P. J., Stereospecificity in meta-fission catabolic pathways. *J. Bacteriol.*, **155**, 424–426 (1983).
11) Kim, D., Kim, Y. S., Kim, S. K., Kim, S. W., Zylstra, G. J., Kim, Y. M., and Kim, E., Monocyclic aromatic hydrocarbon degradation by *Rhodococcus* sp. strain DK17. *Appl. Environ. Microbiol.*, **68**, 3270–3278 (2002).
12) Sakai, M., Miyauchi, K., Kato, N., Masai, E., and Fukuda, M., 2-Hydroxypenta-2,4-dienoate metabolic pathway genes in a strong polychlorinated biphenyl degrader, *Rhodococcus* sp. strain RHA1. *Appl. Environ. Microbiol.*, **69**, 427–433 (2003).
13) Kataoka, M., Ikemi, M., Morikawa, T., Miyoshi, T., Nishi, K., Wada, M., Yamada, H., and Shimizu, S., Isolation and characterization of D-threonine aldolase, a pyridoxal-5′-phosphate-dependent enzyme from *Arthrobacter* sp. DK-38. *Eur. J. Biochem.*, **248**, 385–393 (1997).
14) Liu, J.-Q., Dairi, T., Itoh, N., Kataoka, M., Shimizu, S., and Yamada, H., A novel metal-activated pyridoxal enzyme with a unique primary structure, low specificity D-threonine aldolase from *Arthrobacter* sp. DK-38. *J. Biol. Chem.*, **273**, 16678–16685 (1998).
15) Kataoka, M., Wada, M., Nishi, K., Yamada, H., and Shimizu, S., Purification and characterization of L-allo-threonine aldolase from *Arthrobacter jandaei* DK-39. *FEMS Microbiol. Lett.*, **151**, 245–248 (1997).
16) Liu, J.-Q., Dairi, T., Itoh, N., Kataoka, M., Shimizu, S., and Yamada, H., L-allo-Threonine aldolase from *Arthrobacter jandaei* DK-39: gene cloning, nucleotide sequencing, and identification of the pyridoxal 5′-phosphate-binding lysine residue by site-direct mutagenesis. *J. Bacteriol.*, **179**, 3555–3560 (1997).