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Cloning, Protein Sequence Clarification, and Substrate Specificity of a Leucine Dehydrogenase from *Bacillus sphaericus* ATCC4525

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Abstract Although an X-ray model sequence of a leucine dehydrogenase from *Bacillus sphaericus* ATCC4525 was reported, the amino acid sequence of this enzyme has not been confirmed. In the current study, this leucine dehydrogenase gene was cloned, sequenced, and over-expressed in *Escherichia coli*, and the protein sequence has been clarified. This leucine dehydrogenase is not identical with that of *B. sphaericus* IFO3525 because there are 16 different amino acid residues between these two proteins. Since the information on the catalytic properties of leucine dehydrogenase from *B. sphaericus* ATCC4525 has been limited, the recombinant enzyme was purified as His-tagged protein and further studied. This enzyme showed activity toward aliphatic substrates for both oxidative deamination and reductive amination and is an effective catalyst for the asymmetric synthesis of α-amino acids from the corresponding α-ketoacids.

Keywords Leucine dehydrogenase · Gene cloning · *Bacillus sphaericus* · Reductive amination · Synthesis of α-amino acids

Introduction

Leucine dehydrogenases (LeuDH, EC 1.4.1.9) natively catalyze the reversible conversion of 4-methyl-2-oxopetanoate to L-leucine. Their broad specificity on aliphatic substrates and

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the flexibility on being mutated into aromatic α-amino acid dehydrogenases [1] promote them to be very conceivable starting enzymes for protein engineering. Among L-leucine dehydrogenases, only that from *Bacillus sphaericus* ATCC4525 has crystal structure available [2]. Since understanding of quaternary structure will greatly facilitate structure-directed enzyme evolution, this leucine dehydrogenase has been chosen as the starting point in our effort to create β-amino acid dehydrogenases via in vitro evolution of α-amino acid dehydrogenases. Because the primary structure of LeuDH from *B. sphaericus* ATCC4525 was not elucidated, its quaternary structure was resolved [2] according to the primary structure of the LeuDHs from *Bacillus stearothermophilus* [3] and *Thermoactinomyces intermedius* [4]. Recently, a structural gene (*leudh*) encoding leucine dehydrogenase from *B. sphaericus* IFO 3525 was cloned into *Escherichia coli* cells and sequenced [5]. About 16% of the amino acid residues of the deduced amino acid sequence were different from the X-ray model sequence of LeuDH from *B. sphaericus* ATCC4525. This might result from the uncertainty in the X-ray analysis and/or the existing difference between LeuDHs of these two strains. To clarify this and further characterize the LeuDH from *B. sphaericus* ATCC4525, we cloned, sequenced, and over-expressed this gene in *E. coli* cells. The analysis of the nucleotide sequence of the gene indicates that LeuDHs of these two strains do not have identical primary structures.

Materials and Methods

Materials

B. sphaericus strain ATCC 4525 was purchased from American Type Culture Collection (ATCC). *Taq* DNA polymerase was from Roche (USA), and a Fast-link DNA ligation kit was from Epicentre (USA). Oxidized form (NAD^+) and reduced form (NADH) of nicotinamide adenine dinucleotide were from BioCatalytics (USA). α-Ketoacids and α-amino acids were purchased from Sigma-Aldrich (USA). Ni–nitrilotriacetic acid (NTA) agarose gel was purchased from Qiagen (USA)

Propagation of the Strain and Isolation of Genomic DNA

B. sphaericus ATCC 4525 was revived at a nutrient Luria–Bertani medium plate at 30 °C for 24 h. A single colony was inoculated in nutrient medium at 37 °C to induce overnight growth. The genomic DNA of *B. sphaericus* was extracted using a DNeasy Tissue Kit (Qiagen) by following the standard procedure.

Cloning and Sequencing of the LeuDH Gene

Degenerated primers (1LEHF1 5′-CCGCGCCATGGARATHTTYAARTAYATGGA-3′ and 1LEHR1 5′-CGGGATCCTTADCKNCCRTTHARDATRTT-3′) were applied to amplify the gene. polymerase chain reaction (PCR) was carried out using *Taq* DNA polymerase (Roche) as follows: The reaction was started at 94 °C (2 min) and thermo-cycled for four times: 94 °C (30 s), 50 °C (50 s), 72 °C (70 s), followed by 36 cycles: 94 °C (30 s), 52 °C (50 s), 72 °C (70 s), with a final extension at 72 °C (7 min). The reaction was carried out in 100 μl reaction volume containing 5 pM of 1LEHF1, 20 pM 1LEHR1, 120 ng genomic DNA, 2.0 mM $MgCl_2$, 0.2 mM dNTP, and 2.5 U of *Taq* DNA polymerase. The PCR

product was cloned into pET15b vector at *Nco*I and *Bam*H I sites. The recombinant plasmid LeuDH gene/pET15b (LEH80) was sequenced by IDT (Integrated DNA Technologies).

Cloning of His-tagged LeuDH Gene

The plasmid LEH80 was used as the template for the PCR reaction. In order to delete the *Nde* I site in the gene, primers LEHF1-NdeIdel 5′-AACGAGATGGCATTCCAACCTA TGTG GCAGCGAAC-3′ and LEHR1-NdeIdel 5′-GTTCGCTGCCACATAGGTTGGA ATGC CATCTCGTT-3′ were applied in the site-directed mutagenesis. Primers LEH-NdeIF2–5′-GGGCATATGGAGATATTCAAGTATATGG-3′ and LEH-XhoIR2–5′-AAACTCGAGGCGGCCGTTCAAAATGTT-3′ were applied to flank the ends of the gene. The PCR reaction was started at 94 °C (2 min) and was cycled for 35 times as follows: 94 °C (45 s), 53 °C (45 s), 68 °C (1 min 20 s), with a final extension at 68 °C (10 min). The reaction was carried out in 100 μl reaction volume containing 0.5 pM of each primer, 80 ng of plasmid DNA, 0.2 mM of dNTP, 1 mM of $MgSO_4$, and 1.25 U of *Pfx* DNA polymerase (Invitrogen). The LeuDH gene was subcloned into pET22b + vector at *Nde* I-*Xho* I sites (plasmid LEH96).

Expression and Purification of His-tagged LeuDH

The His-tagged LeuDH gene (LEH96) was expressed in Rosetta 2(DE3)pLysS cells. The culture was grown at 37 °C. At an optical density (OD_{600}) of 1.0, the enzyme expression was induced with 1 mM isopropylthiogalactoside at 30 °C for 5 h. Cells (3 g, wet weight) were harvested by centrifugation. The cell pellet was suspended with 15 ml of lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 10 mM imidazole, pH 8.0), and the cells were broken using Homogenizer (Avestin). The broken cells were centrifuged at 43,150×*g* for 1 h. The cell-free extract (5 ml) was loaded on the Ni–NTA agarose gel (2 ml of gel slurry) column (1.7×14 cm). The column was washed three times with 4 ml of wash buffer (50 mM NaH_2PO_4, 1 M NaCl, and 20 mM imidazole, pH 6.5) each time and then eluted with a buffer containing 50 mM of NaH_2PO_4, 300 mM of NaCl, and 250 mM of imidazole (pH 8.0, 0.7 ml each time). The fractions containing pure protein were collected and desalted with Vivaspin (10K MWCO) column. The resulting enzyme solution was used for kinetic assay.

Activity Assay and Determination of Kinetic Parameters

The enzyme activity was measured by spectrophotometrically monitoring the production of NADH in the oxidative deamination or the consumption of NADH in the reductive amination at 340 nm (ε=6,220 M^{-1} cm^{-1}). The activity was defined as the number of micromoles of NADH produced (or consumed) in 1 min by 1 mg of enzyme (μmol min^{-1} mg^{-1}). The steady-state kinetic parameters of the enzyme was measured at 25 °C under varied substrate concentrations, ranging from 0.0625 to 2 mM for the amino acids and keto acids. Deamination reaction was performed in 100 mM glycine–KCl–KOH buffer (pH 10) with 0.5 mM initial NAD^+ concentration. Amination reaction was performed in 100 mM sodium carbonate buffer (pH 9) with 0.5 mM initial NADH and 975 mM initial NH_4^+ concentrations. The k_{cat} and K_m values were calculated by the method of Lineweaver–Burk plot [6], and the deviation was determined from three parallel groups of velocity measurement.

Syntheses of Aliphatic L-Amino Acids

Into 30 ml of de-ionized water, NH_4Cl (3 mmol), NAD^+ (10 mg), LeuDH (5 mg), formate dehydrogenase (10 mg), and HCO_2Na (3 mmol) were added. After adjusting the pH to 9 with 1 N NaOH solution, 1 mmol of keto acid substrate was added, and the resulting reaction mixture was incubated at 30 °C for about 12 h. The amino acid product was separated via ion-exchange resin (Amberlite IR-120H) and characterized by comparing the retention time on high-performance liquid chromatography (HPLC) with the authentic standards. The enantiomeric purity of the product was determined by chiral HPLC analysis (column: Chirex3126 from Phenomenex, eluent: 2% $CuSO_4$ aqueous solution/isopropanol= 95/5, flow rate: 1 ml/min).

Results and Discussion

Cloning and Sequence Alignment

The first 47 amino acid residues of leucine dehydrogenase from *B. sphaericus* ATCC4525 were determined by protein sequencing, and other residues were deduced from the X-ray crystal structure analysis [2, 7]. Since the gene context was not available, the degenerated primers for amplifying this gene were designed based on the first seven and the last six amino acid residues. The gene fragment was obtained using these degenerated primers via PCR amplification and then cloned into pET15b vector. The PCR amplification and cloning were repeated once. After DNA sequencing, the two randomly picked colonies (one from each PCR experiment) encoded an identical polypeptide consisting of 364 amino acid residues. The DNA sequences only showed some differences at the degenerated nucleotide positions in primer areas (encoding the first seven amino acids and the last six amino acids of this protein). The amino acid sequence of leucine dehydrogenases of *B. sphaericus* ATCC4525 deduced from the DNA sequencing result (ATCC4525) was aligned with that obtained from the crystal structure (ATCC4525Cry) [2] and that of *B. sphaericus* IFO3525 (IFO3525) [5] (Fig. 1). The residues of the later two sequences, which are different from those of *B. sphaericus* ATCC4525 deduced from the DNA sequencing result, are highlighted in yellow. The sequence alignment shows that there are 48 different amino acid residues between the amino acid sequence deduced from the enzyme gene in our experiment and that obtained from previous X-ray analysis. Among them, 16 residues are in domain I (residues 1~136 and 332~364), which is mostly related to the substrate specificity of leucine dehydrogenases. The majority of difference (32 residues) are located in domain II (residues 137~331). Domain II resembles the classical nucleotide-binding domain of lactate dehydrogenase [2]. The X-ray model sequence was obtained based on a consensus sequence from *B. stearothermophilus* and *T. intermedius* LeuDHs and the best fit to the observed electron density. In addition, the electron density for some residues was indistinguishable from weak electron density features associated with disordered residues. Thus, this protocol inevitably led to sequence uncertainty, which was clarified by the current amino acid sequence deduced from the nucleotide sequence. From Fig. 1, it also can be seen that the leucine dehydrogenases from *B. sphaericus* ATCC4525 and IFO3525 are not identical [5]. There are four and 12 different amino acid residues in domains I and II, respectively.

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1   M E I F K Y M E K Y D Y E Q L V F C Q D E A S G L K A V I A  ATCC4525.pro
1   M E I F K Y M E K Y D Y E Q L V F C Q D E A S G L K A V I A  ATCC4525Cry.pro
1   M E I F K Y M E K Y D Y E Q L V F C Q D E A S G L K A I I A  IFO3525.pro

31  I H D T T L G P A L G G A R M W T Y A S E E N A I E D A L R  ATCC4525.pro
31  I H D T T L G P A L G G A R M W T Y N A E E E A I E D A L R  ATCC4525Cry.pro
31  I H D T T L G P A L G G A R M W T Y A T E E N A I E D A L R  IFO3525.pro

61  L A R G M T Y K N A A A G L N L G G G K T V I I G D P F K D  ATCC4525.pro
61  L A R G M T Y K N A A A G L N L G G G K T V I I G D P F A D  ATCC4525Cry.pro
61  L A R G M T Y K N A A A G L N L G G G K T V I I G D P F K D  IFO3525.pro

91  K N E E M F R A L G R F I Q G L N G R Y I T A E D V G T T V  ATCC4525.pro
91  K N E D M F R A L G R F I Q G L N G R Y I T A E D V G T T V  ATCC4525Cry.pro
91  K N E E M F R A L G R F I Q G L N G R Y I T A E D V G T T V  IFO3525.pro

121 L D M D L I H E E T T Y V T G I S P A F G S S G N P S P V T  ATCC4525.pro
121 D D M D L I H Q E T D Y V T G I S P A F G S S G N P S P V T  ATCC4525Cry.pro
121 T D M D L I H E E T N Y V T G I S P A F G S S G N P S P V T  IFO3525.pro

151 A Y G V Y R G M K A A A K E A F G S D S L E G L K V S V Q G  ATCC4525.pro
151 A Y G V Y R G M K A A A K E A F G S D S L E G L A V S V Q G  ATCC4525Cry.pro
151 A Y G V Y R G M K A A A K E A F G T D M L E G R T I S V Q G  IFO3525.pro

181 L G N V A Y K L C E Y L H N E G A K L V V T D I N Q A A I D  ATCC4525.pro
181 L G N V A K A L C K K L N T E G A K L V V T D V N K A A V S  ATCC4525Cry.pro
181 L G N V A Y K L C E Y L H N E G A K L V V T D I N Q A A I D  IFO3525.pro

211 R V V N D F D A I A V A P D E I Y A Q E V D I F S P C A L G  ATCC4525.pro
211 A A V A E E G A D A V A P N A I Y G V T C D I F A P C A L G  ATCC4525Cry.pro
211 R V V N D F G A T A V A P D E I Y S Q E V D I F S P C A L G  IFO3525.pro

241 A I L N D E T I P Q L K A K V I A G S A N N Q L K D S R H G  ATCC4525.pro
241 A V L N D F T I P Q L K A K V I A G S A D N Q L K D P R H G  ATCC4525Cry.pro
241 A I L N D E T I P Q L K A K V I A G S A N N Q L Q D S R H G  IFO3525.pro

271 D Y L H E L G I V Y A P D Y V I N A G G V I N V A D E L Y G  ATCC4525.pro
271 K Y L H E L G I V Y A P D Y V I N A G G V I N V A D E L Y G  ATCC4525Cry.pro
271 D Y L H E L G I V Y A P D Y V I N A G G V I N V A D E L Y G  IFO3525.pro

301 Y N R E R A M K R V D G I Y D S I E K I F A I S K R D G I P  ATCC4525.pro
301 Y N R T R A M K R V D G I Y D S I E K I F A I S K R D G V P  ATCC4525Cry.pro
301 Y N R E R A L K R V D G I Y D S I E K I F E I S K R D S I P  IFO3525.pro

331 T Y V A A N R L A E E R I A R V A K S R S Q F L K N E K N I  ATCC4525.pro
331 S Y V A A D R M A E E R I A K V A K A R S Q F L Q D Q R N I  ATCC4525Cry.pro
331 T Y V A A N R L A E E R I A R V A K S R S Q F L K N E K N I  IFO3525.pro

361 L N G R                                                   ATCC4525.pro
361 L N G R                                                   ATCC4525Cry.pro
361 L N G R                                                   IFO3525.pro
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Fig. 1 Amino acid sequence alignment of leucine dehydrogenases of *B. sphaericus* ATCC4525 deduced from the DNA sequencing result (ATCC4525), obtained from the crystal structure (ATCC4525Cry) and of *B. sphaericus* IFO3525 (IFO3525). The residues different from leucine dehydrogenases of *B. sphaericus* ATCC4525 deduced from the DNA sequencing result are *highlighted in yellow*

Enzyme Purification of His-tagged LeuDH

In order to facilitate the purification of LeuDH protein, the LeuDH gene was subcloned into pET22b + vector to link a six-His tag at the C terminus of protein. The His-tagged LeuDH gene was expressed in *E. coli* cells, and the encoded protein was purified on a Ni–NTA agarose column. sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) showed that the purified enzyme had >90% purity (Fig. 2). The specific activity was increased nearly two times by the purification. The activity yield was 36% (Table 1). The activity of the purified enzyme solution was barely changed after being kept without any stabilizer at 4 °C for a month, indicating that the enzyme was highly stable.

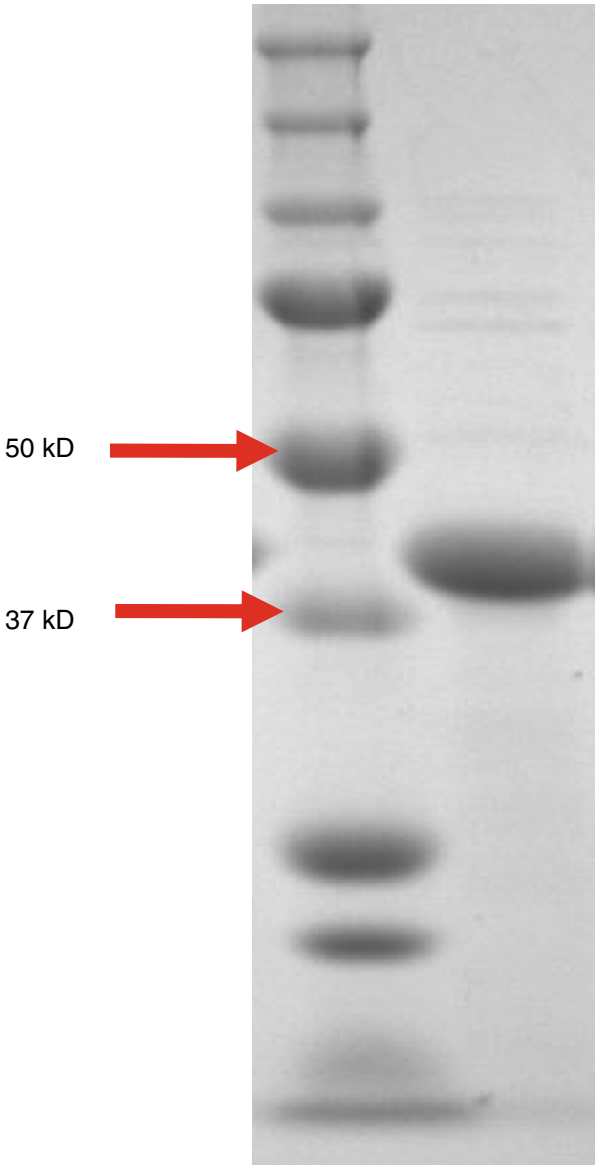

Fig. 2 SDS-PAGE of purified His-tagged LeuDH from *B. sphaericus*. *Left lane*, Protein marker; *right lane*, purified enzyme

Table 1 Purification of His-tagged LeuDH by Ni–NTA agarose column.

	Total protein (mg)	Total activity (U)[a]	Specific activity (U/mg)[a]	Yield (%)
Cell-free extract	354.24	2,222.50	6.27	100
After Ni column	65.61	799.98	12.19	36

[a] The activity unit (U) was defined as 1 μmol of NADH consumed in 1 min (μmol min^{-1}) using L-leucine as substrate.

Table 2 Kinetic parameters of His-tagged LeuDH for both oxidative deamination and reductive amination.

Substrates	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m
L-Leucine	10.83±0.97	1.12±0.21	9.54
L-Isoleucine	3.31±0.40	0.78±0.07	4.17
L-Norvaline	1.53±0.13	0.98±0.01	1.54
L-Norleucine	0.18±0.03	1.27±0.25	0.14
4-Methyl-2-oxopentanoic acid	99.38±1.70	1.55±0.14	58.53
3-Methyl-2-oxopentanoic acid	27.63±1.16	0.38±0.07	71.29
2-Oxopentanoic acid	47.69±5.71	1.08±0.11	43.59

Kinetic Parameters of His-tagged LeuDH

The kinetic parameters of leucine dehydrogenase from *B. sphaericus* ATCC4525 for the oxidative deamination of L-leucine, L-isoleucine, and L-norvaline and the reductive amination of their corresponding α-ketoacids were determined. The results are presented in Table 2. The K_m values for L-amino acids are consistent with those reported for leucine dehydrogenases from other strains including *B. stearothermophilus* [3], *B. sphaericus* IFO3525[8], *Natronobacterium magadii* MS-3 [9]. The catalytic constant k_{cat} and specificity constant k_{cat}/K_m for the reductive amination are much higher than those for the reverse oxidative deamination, suggesting this leucine dehydrogenase would be an effective catalyst for the synthesis of α-amino acids from the corresponding α-ketoacids.

Substrate Specificity of LeuDH

The substrate specificity of leucine dehydrogenase from *B. sphaericus* ATCC4525 was studied for both the reductive amination and oxidative deamination (Tables 3 and 4). Among the eight ketoacids tested, the enzyme showed activity toward all the aliphatic α-keto acids; no activity was observed for the three aryl α-ketoacids (Table 3). This is consistent with the observations for other known leucine dehydrogenases. The structure of aliphatic α-keto acids greatly affected the enzyme activity (Table 3). From Table 4, it can be seen that, in addition to the native substrate L-leucine, LeuDH is active toward other aliphatic α-amino acids. However, the length and the structure of branch groups exert significant effect on the enzyme activity.

Table 3 Relative activity of LeuDH toward the reductive amination of α-ketoacids.

Entry	Compounds	Relative activity
1	4-Methyl-2-oxopentanoic acid	100[a]
2	3-Methyl-2-oxopentanoic acid	64
3	2-Oxopentanoic acid	25
4	4-Methylthio-2-oxobutyric acid	19
5	2-Oxobutyric acid	11
6	2-Oxo-3-phenylpropionic acid	0
7	3-(2′-Nitrophenyl)-2-oxopropionic acid	0
8	3-(3′-Indolyl)-2-oxopropionic acid	0

[a] The specific activity for 4-methyl-2-oxopentanoic acid was 39.35 μmol·min^{-1} mg^{-1}.

Table 4 Relative activity of LeuDH toward the oxidative deamination of α-amino acids.

Entry	Compounds	Relative activity
1	L-Leucine	100[a]
2	L-Isoleucine	72.4
3	L-Norvaline	36.8
4	L-2-Aminobutyric acid	5.7
5	L-Alanine	3.4
6	L-Methionine	1.2
7	L-Cysteine	0
8	L-Phenylalanine	0

[a] The specific activity for L-leucine was 12.19 μmol·min^{-1} mg^{-1}.

Synthesis of Aliphatic L-Amino Acids

Since the above kinetic parameters suggested that leucine dehydrogenase from *B. sphaericus* ATCC4525 would be an effective catalyst for the reductive amination of aliphatic α-ketoacids to the corresponding α-amino acids, several aliphatic α-ketoacids were tested at a preparative scale. The reductive amination reactions were carried out at 30 °C, and the products were isolated by ion-exchange chromatography. The isolated yield and enantiomeric excess of the product α-amino acids are summarized in Table 5. All the α-keto acids were completely converted to the corresponding L-amino acids in essentially optically pure form. For 3-methyl-2-oxopetanoic acid, a mixture of equal amount of L-isoleucine and L-allo-isoleucine were obtained, indicating leucine dehydrogenase from *B. sphaericus* ATCC4525 could not differentiate the chiral center at the β-position.

In conclusion, a leucine dehydrogenase gene from *B. sphaericus* ATCC4525 was cloned, sequenced and over-expressed in *E. coli*. The amino acid sequence alignment of encoded protein reveals that 48 amino acid residues are different from those in the sequence obtained from X-ray crystal analysis of LeuDH from same strain, and 16 amino acid residues are distinct from that of LeuDH from *B. sphaericus* IFO3525. This study clarified the amino acids sequence of leucine dehydrogenase from *B. sphaericus* ATCC4525 and showed that it is not identical with that of *B. sphaericus* IFO3525. The recombinant enzyme was purified as His-tagged protein, which showed activity toward aliphatic substrates for both oxidative deamination and reductive amination. As demonstrated in Table 5, this enzyme is an effective catalyst for the asymmetric synthesis of α-amino acids from the corresponding α-ketoacids.

Table 5 LeuDH-catalyzed synthesis of aliphatic L-amino acids.

Substrate	Product	Yield (%)	ee (%)
4-Methyl-2-oxopetanoic acid	L-Leucine	91	>99
3-Methyl-2-oxopetanoic acid	L-Isoleucine/l-allo-isoleucine	92	>99[a]
2-Oxobutanoic acid	L-2-Aminobutyric acid	89	>99
2-Oxopentanoic acid	L-Norvaline	78	>99

[a] The ratio of L-isoleucine to L-allo-isoleucine is 1:1, the ee values of both diastereomers were >99%.

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