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Cloning and sequencing of the leucine dehydrogenase gene from *Bacillus sphaericus* IFO 3525 and importance of the C-terminal region for the enzyme activity

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Dedicated to Professor Dr. Kenji Soda in honor of his 70th birthday

Abstract

The structural gene (*leudh*) coding for leucine dehydrogenase from *Bacillus sphaericus* IFO 3525 was cloned into *Escherichia coli* cells and sequenced. The open reading frame coded for a protein of 39.8 kDa. The deduced amino acid sequence of the leucine dehydrogenase from *B. sphaericus* showed 76–79% identity with those of leucine dehydrogenases from other sources. About 16% of the amino acid residues of the deduced amino acid sequence were different from the sequence obtained by X-ray analysis of the *B. sphaericus* enzyme. The recombinant enzyme was purified to homogeneity with a 79% yield. The enzyme was a homooctamer (340 kDa) and showed the activity of 71.7 μmol·min^{-1}·mg^{-1}) of protein. The mutant enzymes, in which more than six amino acid residues were deleted from the C-terminal of the enzyme, showed no activity. The mutant enzyme with deletion of four amino acid residues from the C-terminal of the enzyme was a dimer and showed 4.5% of the activity of the native enzyme. The dimeric enzyme was more unstable than the native enzyme, and the K_m values for L-leucine and NAD$^+$ increased. These results suggest that the Asn-Ile-Leu-Asn residues of the C-terminal region of the enzyme play an important role in the subunit interaction of the enzyme.

Keywords: Leucine dehydrogenase; *Bacillus sphaericus*; Primary structure; Nucleotide sequence; Subunit interaction

1. Introduction

Leucine dehydrogenase (L-leucine:NAD$^+$ oxidoreductase, deaminating, EC 1.4.1.9) catalyzes the reversible deamination of L-leucine and several other branched-chain and straight-chain L-amino acids to their keto analogs (Fig. 1). The enzyme occurs in endospore-forming bacteria such as bacilli [1,2] and clostridia [3] and a non-spore-forming bacterium *Corynebacterium pseudodiphtheriticum* [1,4]. The enzyme was purified and characterized from *Bacillus subtilis* [5], *Bacillus sphaericus* [1], *Bacillus cereus* [6], *Bacillus stearothermophilus* [7], *Bacillus caldolyticus* [8], *Bacillus licheniformis* [9], *Bacillus* sp. DSM 730 [10], *Clostridium thermoaceticum* [3], *C. pseudodiphtheriticum* [4], and *Thermoactinomyces intermedius* [11]. The enzyme is useful for the industrial synthesis of branched-chain L-amino acids and the analyses of branched-chain L-amino acids, their keto analogs, and the activity of serum leucine aminopeptidase [12–14]. The enzyme gene (*leudh*) was cloned into *Escherichia coli* cells to obtain large

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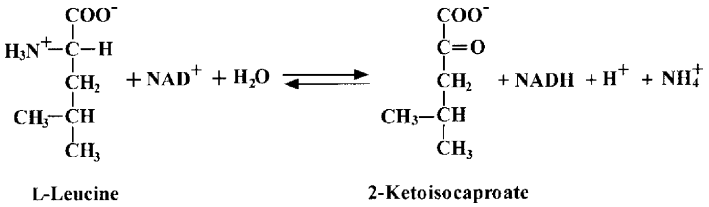

Fig. 1. Leucine dehydrogenase reaction.

amounts of the purified enzyme as well as to study the structure and function relationship of the enzyme [3,9–11,15,16]. The three-dimensional structure of the *B. sphaericus* enzyme was analyzed [17] according to the primary structure of the *B. stearothermophilus* [15] and *T. intermedius* [11] enzymes, since the primary structure of the *B. sphaericus* enzyme was not elucidated. To confirm the primary structure of the *B. sphaericus* enzyme and to use the enzyme for the synthesis of branched-chain L-amino acids, we cloned the *leudh* gene of *B. sphaericus* into *E. coli* cells and analyzed the nucleotide sequence of the gene. We also investigated the role of the C-terminal region in the enzyme activity by deletion analysis.

In this paper, we describe the cloning, sequencing, and overexpression in *E. coli* cells of the *leudh* gene and the deletion of the C-terminal region of the recombinant enzyme.

2. Experimental

2.1. Materials

NAD^+ and NADH were purchased from Kohjin Biochemicals (Tokyo, Japan); a TSK gel G3000SW column from Tosoh (Tokyo, Japan); marker proteins for molecular mass measurement from Oriental Yeast (Osaka, Japan); oligonucleotides for polymerase chain reaction (PCR) from Hokkaido System Science (Hokkaido, Japan); and Ex Taq DNA polymerase and an LA PCR in vitro cloning kit from Takara Shuzo (Kyoto, Japan). Other chemicals were of analytical grade.

2.2. Microorganisms and culture conditions

B. sphaericus IFO 3525, which was used as a source of chromosomal DNA, was grown aerobically at 30 °C for 20 h in a peptone medium (1% peptone, 0.2% K_2HPO_4, 0.2% KH_2PO_4, 0.2% NaCl, 0.01% $MgSO_4 \cdot 7H_2O$, and 0.01% yeast extract, pH 7.2). *E. coli* clones were grown aerobically at 37 °C for 20 h in 100 ml of a Luria-Bertani (LB) medium (1% peptone, 0.5% yeast extract, and 0.5% NaCl, pH 7.2) containing ampicillin (50 μg/ml) and isopropyl-β-D-thiogalactopyranoside (100 μg/ml).

2.3. Cloning and sequencing of the leudh gene

The chromosomal DNA of *B. sphaericus* was prepared by the method of Saito and Miura [18]. Sense (S) and antisense (A) primers were designed on the basis of the amino acid sequences conserved in the *B. stearothermophilus* [15] and *T. intermedius* [11] enzymes. The sequences were 5′-GGGAATTCTAYGAYTAYGARCAR-3′ (primer S containing an underlined *Eco*RI site) and 5′-GGGGATCCTCRTCTGCTACRTTDATNAC-3′ (primer A containing an underlined *Bam*HI site). PCR was done with Ex Taq DNA polymerase. The amplified DNA fragments (890 bp) were ligated into the *Eco*RI–*Bam*HI site of pUC18 and introduced into *E. coli* JM109 cells. The nucleotide sequence of the cloned PCR product was analyzed with an Applied Biosystems 373A DNA sequencer and a DNA sequencing kit (Perkin-Elmer, Boston, MA, USA). From the sequence obtained, two antisense primers, R0 (5′-GTCGTGAATCTTGTAGTTGG-3′) and R1 (5′-CCAGATGCTTCGTCTTGG-3′), and two sense primers, F1 (5′-CAGCATTAGGTGGTGCTCGTATGTGGACCTAC-3′) and F2 (5′-TATTTACACGAGCTAGGC-3′), were designed. For the sequencing of unknown DNA regions at the 5′- and 3′-ends of the *leudh* gene, cassette-ligation-mediated PCR [19] was performed using an LA PCR in vitro cloning kit. Finally, the *leudh* gene was amplified by PCR with Ex Taq DNA polymerase and with the

primers of BS-ECO containing an *Eco*RI site and BS-SMA containing a *Sma*I site. The sequences were 5′-GGGAATTCATGGAAATCTTCAAGTATATGG-3′ (a sense primer BS-ECO) and 5′ -CCGGGCCC-AATAGACGTTGTTAACGGCCGTTCAAAA-3′ (an antisense primer BS-SMA). The amplified fragment (1.1 kb) was ligated into the *Eco*RI-*Sma*I site of pUC18. We designated the constructed plasmid pUB-SLEU. The sequence of the *leudh* gene in the plasmid was determined in both directions as described above. The nucleotide sequence data will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases under the accession number AB103119.

To obtain a high-expression strain, the *Eco*RI-*Sma*I fragment (1.1 kb) was ligated into the *Eco*RI-*Sma*I site of pKK223-3 and the constructed plasmid was designated as pKBSLEU.

The mutant enzymes, in which 1–14 amino acid residues from the C-terminal of the enzyme were deleted, were obtained by the PCR method. PCR was done with Ex Taq DNA polymerase with the sense primer, BS-ECO, and the following antisense primers with an underlined *Sma*I site: DEL-1, DEL-2, DEL-4, DEL-6, DEL-7, DEL-9, and DEL-14. The sequences were 5′-GGCCCGGGTTAGCCGTTCA-AAATATTTTTTTCATTTTTTAAG-3′ (DEL-1), 5′-GGCCCGGGTTAGTTCAAAATATTTTTTTCATTT-TTTAAGAAC-3′ (DEL-2), 5′-GGCCCGGGTTAAA-TATTTTTTTCATTTTTTAAGAACTGAC-3′ (DEL-4), 5′-G-GCCCGGGTGGTTATTTTTCATTTTTTA-AGAACTGACTACGC-3′ (DEL-6), 5′-GGCCCGGG-TGGTTATTCATTTTTTAAGAACTGACTACGCG-A-3′ (DEL-7), 5′-GGCCCGGGTTATTTTAAGAAC-TGACTACGCGATTTCGC-3′ (DEL-9), and 5′-GG-CCCGGGTTAACGCGATTTCGCTACACGAGCA-ATACG-3′ (DEL-14). The amplified fragments were ligated into the *Eco*RI-*Sma*I site of pKK223-3 and the constructed plasmids were named pKBSDEL-1, pKBSDEL-2, pKBSDEL-4, pKBSDEL-6, pKBSDEL-7, pKBSDEL-9, and pKBSDEL-14, respectively.

The *leudh* gene from *B. stearothermophilus* IFO 12550 was cloned into *E. coli* JM109 cells as described in the paper [15] and sequenced.

2.4. Enzyme and protein assays

The standard reaction mixture contained 10 μmol of L-leucine, 5 μmol of NAD^+, 200 μmol of glycine–KCl–KOH buffer (pH 10.5), and enzyme in a final volume of 1.0 ml. The substrate was replaced with water in a blank. Incubation was carried out at 30 °C in a cuvette with a 1 cm light path. The reaction was started by addition of NAD^+ and monitored by measuring the initial change in absorbance at 340 nm with a Shimadzu UV-140-02 spectrophotometer. One unit of enzyme was defined as the amount that catalyzed the formation of 1 μmol of NADH per minute in the oxidative deamination with a molar absorption coefficient of 6220 M^{-1} cm^{-1}. Specific activity was expressed as units per milligram of protein. Protein was measured by the method of Lowry et al. [20], with egg albumin as the standard.

2.5. Purification of the enzyme

All purification procedures were performed at 0–5 °C, and potassium phosphate buffer (pH 7.2) containing 0.01% 2-mercaptoethanol was used in the procedures unless otherwise stated.

The cells of *E. coli* JM109/pKBSLEU (2.6 g, wet weight) were suspended in 10.2 ml of 0.1 M buffer and disrupted by sonication at 4 °C. The supernatant obtained by centrifugation at 10,000 × *g* for 1 h was dialyzed against 2 l of 10 mM buffer at 4 °C overnight, and the dialyzed solution was used as the cell extract. The cell extract (258 mg) was applied to a DEAE-Toyopearl column (1.1 cm × 11 cm) equilibrated with 10 mM buffer. After the column had been washed with the same buffer and the buffer containing 0.1 M KCl, the enzyme was eluted with the buffer containing 0.2 M KCl. The active fractions were concentrated with an Amicon ultrafiltration unit with a PM-10 membrane filter and dialyzed against 10 mM buffer overnight.

2.6. Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with 10% polyacrylamide gel by the method of Laemmli [21].

2.7. Determination of molecular mass

The molecular mass was determined at room temperature by high-pressure liquid chromatography on a TSK gel G3000SW column (0.75 cm × 60 cm) at a

flow rate of 0.7 ml/min with 0.1 M potassium phosphate buffer (pH 7.0) containing 0.2 M NaCl [22]. The molecular mass of the subunit was estimated by SDS-PAGE with the following standard proteins (Pharmacia, Uppsala, Sweden): catalase (60 kDa), ovalbumin (45 kDa), yeast alcohol dehydrogenase (37 kDa), α-chymotrypsinogen A (25 kDa), and myoglobin (17.2 kDa).

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CAAGAAAGCCTCCGGAATCTAAAGGCGTCTCGTGACAAGCGCGGGAAGAAGCTCAAGAAACTAGTAATCGTA  -192
ACTGAATTTTTAGAGAGAAATTCTTACTTAGTAGGAAAAGTGTAATAATAGTTGCGAAAATTGTGAATTTGT  -120
CAGTATTGTAAAATTAAATGGAGCGATATTATCCATTGCAAAACAGTTTTATCATTTTTTTAAAATTTGATT   -48
GGATGGGCATTAGGGGTTGCACTTTCCAAAGCAAAGGGGCGAGTTTTCATGGAAATCTTCAAGTATATGGAA    24
                                                  M  E  I  F  K  Y  M  E      8
AAGTATGATTATGAACAATTGGTATTTTGCCAAGACGAAGCATCTGGGTTAAAAGCGATTATCGCTATCCAT    96
 K  Y  D  Y  E  Q  L  V  F  C  Q  D  E  A  S  G  L  K  A  I  I  A  I  H     32
GACACAACACTTGGACCAGCATTAGGTGGTGCTCGTATGTGGACCTACGCGACAGAAGAAAATGCGATTGAG   168
 D  T  T  L  G  P  A  L  G  G  A  R  M  W  T  Y  A  T  E  E  N  A  I  E     56
GATGCATTAAGATTAGCACGCGGGATGACATATAAAAATGCAGCTGCTGGTTTAAACCTTGGCGGTGGAAAA   240
 D  A  L  R  L  A  R  G  M  T  Y  K  N  A  A  A  G  L  N  L  G  G  G  K     80
ACGGTCATTATTGGGGACCCATTTAAAGATAAAAACGAAGAAATGTTCCGTGCTTTAGGTCGTTTCATTCAA   312
 T  V  I  I  G  D  P  F  K  D  K  N  E  E  M  F  R  A  L  G  R  F  I  Q    104
GGATTAAACGGTCGCTATATTACCGCTGAAGATGTTGGTACAACCGTAACAGATATGGATTTAATCCATGAG   384
 G  L  N  G  R  Y  I  T  A  E  D  V  G  T  T  V  T  D  M  D  L  I  H  E    128
GAAACAAATTACGTTACAGGTATATCGCCAGCGTTTGGTTCATCGGGTAATCCTTCACCAGTAACTGCTTAT   456
 E  T  N  Y  V  T  G  I  S  P  A  F  G  S  S  G  N  P  S  P  V  T  A  Y    152
GGCGTTTATCGTGGCATGAAAGCAGCGGCGAAAGAAGCATTTGGTACGGATATGCTAGAAGGTCGTACTATA   528
 G  V  Y  R  G  M  K  A  A  A  K  E  A  F  G  T  D  M  L  E  G  R  T  I    176
TCGGTACAAGGGCTAGGAAACGTAGCTTACAAGCTTTGCGAGTATTTACATAATGAAGGTGCAAAACTTGTA   600
 S  V  Q  G  L  G  N  V  A  Y  K  L  C  E  Y  L  H  N  E  G  A  K  L  V    200
GTAACAGATATTAACCAAGCGGCTATTGATCGTGTTGTCAATGATTTTGGCGCTACAGCAGTTGCACCTGAT   672
 V  T  D  I  N  Q  A  A  I  D  R  V  V  N  D  F  G  A  T  A  V  A  P  D    224
GAAATCTATTCACAAGAAGTCGATATTTTCTCACCGTGTGCACTTGGCGCAATTTTAAATGACGAAACGATT   744
 E  I  Y  S  Q  E  V  D  I  F  S  P  C  A  L  G  A  I  L  N  D  E  T  I    248
CCGCAATTAAAAGCAAAAGTTATTGCTGGTTCTGCTAATAACCAACTACAAGATTCACGACATGGAGATTAT   816
 P  Q  L  K  A  K  V  I  A  G  S  A  N  N  Q  L  Q  D  S  R  H  G  D  Y    272
TTACACGAGCTAGGCATTGTTTATGCACCTGACTATGTCATTAATGCAGGTGGTGTAATAAATGTCGCGGAC   888
 L  H  E  L  G  I  V  Y  A  P  D  Y  V  I  N  A  G  G  V  I  N  V  A  D    296
GAATTATATGGCTATAATCGTGAACGAGCGTTGAAACGTGTAGATGGTATTTACGATAGTATTGAAAAAATC   960
 E  L  Y  G  Y  N  R  E  R  A  L  K  R  V  D  G  I  Y  D  S  I  E  K  I    320
TTTGAAATTTCCAAACGTGATAGTATTCCAACATATGTTGCGGCAAATCGTTTGGCAGAAGAACGTATTGCT  1032
 F  E  I  S  K  R  D  S  I  P  T  Y  V  A  A  N  R  L  A  E  E  R  I  A    344
CGTGTAGCGAAATCGCGTAGTCAGTTCTTAAAAAATGAAAAAAATATTTTGAACGGCCGTTAACAACGTCTA  1104
 R  V  A  K  S  R  S  Q  F  L  K  N  E  K  N  I  L  N  G  R  *             364
TTCGCTACAGAGAAGTCGCATCGTCCGACTCCCT                                         1138
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Fig. 2. Nucleotide sequence of the *leudh* gene from *B. sphaericus* IFO 3525 and the deduced amino acid sequence. Initiation and termination codons are underlined.

2.8. Analyses of the N-terminal and C-terminal amino acid sequences

The N-terminal amino acid analysis of the enzyme was done by automated Edman degradation with an Applied Biosystems 492 protein sequencer. The phenylthiohydantoin amino acid derivatives were identified with an Applied Biosystems model 120A phenylthiohydantoin derivative on-line analyzer. The C-terminal peptide was obtained with a Shimadzu CTFF-1 automatic C-terminal fragment fractionator, after digestion of the enzyme with lysyl endopeptidase [23].

3. Results and discussion

3.1. Cloning and nucleotide sequence of the leudh gene

We cloned the *leudh* gene from *B. sphaericus* into *E. coli* JM109 cells and analyzed the entire nucleotide sequence of the gene as described in the Section 2. The nucleotide sequence of the *Eco*RI-*Sma*I fragment (1.1 kb) had an open reading frame encoding the enzyme (Fig. 2). The gene encoded a polypeptide consisting of 364 amino acid residues. The calculated molecular mass of this protein was 39,829 Da.

3.2. Overproduction and purification of the enzyme

The *E. coli* JM109 cells harboring the plasmid pUBSLEU showed the enzyme activity (0.066 U/mg of protein). The *E. coli* JM109 cells harboring the plasmid pKBSLEU produced a high level of the enzyme (10.6 U/mg of protein). This activity was 25-fold higher than that of the crude extract of *B. sphaericus* IFO 3525. The enzyme was purified to homogeneity from the cell extracts of *E. coli* JM109/pKBSLEU by DEAE-Toyopearl 650 column chromatography with a 79% yield. The purified recombinant enzyme gave a single band on SDS-PAGE (Fig. 3) and showed the activity of 71.7 U/mg of protein.

3.3. Molecular mass and subunit structure

The molecular mass of the recombinant enzyme was estimated to be 340 kDa by gel filtration on a TSK gel G3000SW column. The molecular mass of the subunit was calculated to be 43 kDa by SDS-PAGE. These results suggest that the enzyme consists of eight identical subunits. The first 20 N-terminal and the 5 C-terminal amino acid sequences are in good agreement with that deduced from the nucleotide sequence of the *leudh* gene.

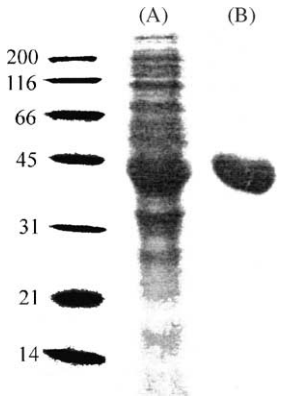

Fig. 3. SDS-PAGE of the crude extract of transformed *E. coli* cells and the purified recombinant enzyme. Lane A, crude extract of the *E. coli* JM109 harboring pKBSLEU and lane B, the enzyme purified by DEAE-Toyopearl column chromatography.

3.4. Alignment of the amino acid sequences of leucine dehydrogenases

The amino acid sequence of the *B. sphaericus* enzyme was similar to those of leucine dehydrogenases from other sources (Fig. 4). Percentages of identical amino acids of the enzyme compared with the enzymes from *B. cereus* [16], *B. licheniformis* [9], *Bacillus* sp. DSM 730 (DDBJ/EMBL/GenBank accession no. AB103386), *B. stearothermophilus* (accession no. AB103384), *C. thermoaceticum* (accession no. AB103385), and *T. intermedius* [11] were estimated to be 79, 79, 76, 76, 76, and 77%, respectively. The catalytically important lysine residues (Lys68 and Lys80) [17,24–26] and the common GXGXXA(G) sequence, which is characteristic of a NAD^+-binding site [27] are highly conserved in these enzymes. The amino acid sequence of the *B. stearothermophilus* enzyme [15] was 62 amino acid residues longer than the other leucine dehydrogenases. Thus, we analyzed nucleotide sequence of a DNA fragment containing the *leudh* gene of *B. stearothermophilus*.

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BS   M--EIFKYMEKYDYEQLVFCQDEASGLKAIIAIHDTTLGPALGGARMWTYATEENAIEDA  58
BC   MTLEIFEYLEKYDYEQVVFCQDKESGLKAIIAIHDTTLGPALGGTRMWTYDSEEAAIEDA  60
BL   M--ELFRYMEQYDYEQLVFCQDKQSGLKAIIAIHDTTLGPALGGTRMWTYESEEAAIEDA  58
BD   M--ELFKYMEMYDYEQVLFWQDKESGLKAIIAIHDTTLGPALGGTRMWMYNSEEEALEDA  58
BT   M--ELFKYMETYDYEQVLFCQDKESGLRAIIAIHDTTLGPALGGTRMWMYNSEEEALGDA  58
CT   M--ELFKYMETYDYEQVLFCQDKESGLRAIIAIHDTTLGPALGGTRMWMYNSEEEALGDA  58
TI   M--KIFDYMEKYDYEQLVMCQDKESGLKAIICIHVTTLGPALGGMRMWTYASEEEAIEDA  58
     *    * * * *****    **  *** *** ** ********* *** *  ** *  **

BS   LRLARGMTYKNAAAGLNLGGGKTVIIGDPFKDKNEEMFRALGRFIQGLNGRYITAEDVGT  118
BC   LRLAKGMTYKNAAAGLNLGGAKTVIIGDPRKDKSEAMFRALGRYIQGLNGRYITAEDVGT  120
BL   LRLARGMTYKNAAAGLNLGGGKTVIIGDPRKDKNEEMFRAFGRYIQGLNGRYITAEDVGT  118
BD   LRLARGMTYKNAAAGLNLGGGKTVIIGDPRKDKNEAMFRAFGRFIQGLNGRYITAEDVGT  118
BT   LRLARGMTYRNTAAGLNLGGGKTVIIGDPRKDKNEAMFRAFGRFIQGLNGRYITAEDVGT  118
CT   LRLARGMTYRNTAAGLNLGGGKTVIIGDPRKDKNEAMFRAFGRFIQGLNGRYITAEDVGT  118
TI   LRLGRGMTYKNAAAGLNLGGGKTVIIGDPRKDKNEAMFRALGRFIQGLNGRYITAEDVGT  118
     ***  **** * ******** ******** *** * **** ** ****************

BS   TVTDMDLIHEETNYVTGISPAFGSSGNPSPVTAYGVYRGMKAAAKEAFGTDMLEGRTISV  178
BC   TVDDMDIIHEETDFVTGISPSFGSSGNPSPVTAYGVYRGMKAAAKEAFGTDNLEGKVIAV  180
BL   TVEDMDIIHDETDFVTGISPAFGSSGNPSPVTAYGVYKGMKAAAKAAFGTDSLEGKTVAV  178
BD   TVADMDIIYHETDYVTGISPEFGSSGNPSPATAYGVYRGMKAAAKEAFGSDSLEGKVVAV  178
BT   TVADMDIIYQETDYVTGISPEFGSSGNPSPATAYGVYRGMKAAAKEAFGSDSLEGKVVAV  178
CT   TVADMDIIYQETDYVTGISPEFGSSGNPSPATAYGVYRGMKAAAKEAFGSDSLEGKVVAV  178
TI   TVEDMDIIHEETRYVTGVSPAFGSSGNPSPVTAYGVYRGMKAAAKEAFGDDSLEGKVVAV  178
     ** *** *  **  *** ** ********* ****** ******* *** * ***    *

BS   QGLGNVAYKLCEYLHNEGAKLVVTDINQAAIDRVVNDFGATAVAPDEIYSQEVDIFSPCA  238
BC   QGVGNVAYHLCKHLHAEGAKLIVTDINKEAVQRAVEEFGASAVEPNEIYGVECDIYAPCA  240
BL   QGVGNVAYNLCRHLHEEGAKLIVTDINKEAVERAVAEFGARAVDPDDIYSQECDIYAPCA  238
BD   QGVGNVAYHLCRHLHEEGAKLIVTDINKEAVARAVEEFGAKAVDPNDIYGVECDIFAPCA  238
BT   QGVGNVAYHLCRHLHEEGAKLIVTDINKEAVARAVEEFGAKAVDPNDIYGVECDIFAPCA  238
CT   QGVGNVAYHLCRHLHEEGAKLIVTDINKEAVARAVEEFGAKAVDPNDIYGVECDIFAPCA  238
TI   QGVGHVAYELCKHLHNEGAKLIVTDINKENADRAVQEFGAEFVHPDKIYDVECDIFAPCA  238
     ** * *** **  ** ***** *****     * *  ***  * *  **  * **  ***

BS   LGAILNDETIPQLKAKVIAGSANNQLQDSRHGDYLHELGIVYAPDYVINAGGVINVADEL  298
BC   LGATVNDETIPQLKAKVIAGSANNQLKEDRHGDIIHEMGIVYAPDYVINAGGVINVADEL  300
BL   LGATINDDTIPQLKAKVIAGAANNQLKETRHGDQIHDMGIVYAPDYVINAGGVINVADEL  298
BD   LGGIINDHTIPQLKAKVIAGSVNNQLKEPRHGDMIHEMGIVYAPDYVINAGGVINVADEL  298
BT   LGGIINDQTIPQLKAKVIAGSANNQLKEPRHGDIIHEMGIVYAPDYVINAGGVINVADEL  298
CT   LGGIINDQTIPQLKAKVIAGSANNQLKEPRHGDIIHEMGIVYAPDYVINAGGVINVADEL  298
TI   LGAIINDETIERLKCKVVAGSANNQLKEERHGKMLEEKGIVYAPDYVINAGGVINVADEL  298
     **   ** **  ** ** **  ****   ***      **********************

BS   YGYNRERALKRVDGIYDSIEKIFEISKRDSIPTYVAANRLAEERIARVAKSRSQFLKNEK  358
BC   YGYNRERALKRVESIYDTIAKVIEISKRDGIATYVAADRLAEERIASLKNSRSTYLRNGH  360
BL   YGYNSERALKKVEGIYGNIERVLEISKRDRIPTYLAADRLAEERIERMRQSRSQFLQNGH  358
BD   YGYNRERSMKMIEQIFDNIEKVFAIAKRDNIPTY-AADRMAEERIETMRKARSQFLQNGH  357
BT   YGYNRERAMKKIEQIYDNIEKVFAIAKRDNIPTYVAADRMAEERIETMRKARSQFLQNGH  358
CT   YGYNRERAMKKIEQIYDNIEKVFAIAKRDNIPTYVAADRMAEERIETMRKARSQFLQNGH  358
TI   LGYNRERAMKKVEGIYDKILKVFEIAKRDGIPSYLAADRMAEERIEMMRKTRSTFLQDQR  358
     **** **  *    *   *     * *** *  * ** * *****      **  *

BS   NILNGR                                                        364
BC   DIISRR                                                        366
BL   HILSRR                                                        364
BD   HILSRRRAR                                                     366
BT   HILSRRRAR                                                     367
CT   HILSRRRAR                                                     367
TI   NLINFNNK                                                      366
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Fig. 4. Alignment of the amino acid sequences of leucine dehydrogenases from *B. sphaericus* (BS), *B. cereus* (BC) [16], *B. licheniormis* (BL) [9], *Bacillus* sp. DSM 730 (BD) (accession no. AB103386), *B. stearothermophilus* (BT) (accession no. AB103384), *C. thermoaceticum* (CT) (accession no. AB103385), and *T. intermedius* (TI) [11]. Asterisks indicate conserved residues in the seven sequences.

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X-ray MEIFKYMEKYDYEQLVFCQDEASGLKAVIAIHDTTLGPALGGARMFTYGAEEEAIEDALRLAR
GENE  MEIFKYMEKYDYEQLVFCQDEASGLKAIIAIHDTTLGPALGGARMWTYATEENAIEDALRLAR

X-ray GMTYKNAAAGLNLGGGKTVIIGDPFADKNEAMFRALGRFIQGLNGRYITAEDVGTTVSDMDLI
GENE  GMTYKNAAAGLNLGGGKTVIIGDPFKDKNEEMFRALGRFIQGLNGRYITAEDVGTTVTDMDLI

X-ray HQETDYVTGISPAFGSSGGPSPVTAYGVYRGMKAAAAEAFGSDSLAGDAVAVQGLGNVAKAL
GENE  HEETNYVTGISPAFGSSGNPSPVTAYGVYRGMKAAAKEAFGTDMLEGRTISVQGLGNVAYKL

X-ray CKKLNTEGAALVVTDVNHGAVSAAVADEGADAAAPNAIYGVTCDIFAPCAVGAVLNDFTI
GENE  CEYLHNEGAKLVVTDINQAAIDRVVNDFGATAVAPDEIYSQEVDIFSPCALGAILNDETI

X-ray PQLAAAVIAGSADNQLKDPRHGKYLHELGIVYAPDYVINAGGVINVADELYGYNRTRAMAKV
GENE  PQLKAKVIAGSANNQLQDSRHGDYLHELGIVYAPDYVINAGGVINVADELYGYNRERALKRV

X-ray EGIYDTIEKIFAIAKRDGVPSYVAADRMAEERIAKVAKARSQFLQDQRNILNGR     364
GENE  DGIYDSIEKIFEISKRDSIPTYVAANRLAEERIARVAKSRSQFLKNEKNILNGR     364
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Fig. 5. Comparison of the amino acid sequence deduced from the nucleotide sequence of the *leudh* gene (GENE) with that obtained by X-ray analysis (X-ray) of the leucine dehydrogenase from *B. sphaericus*. Different residues in two sequences were shown in boldface type.

The *leudh* gene encoded 367 amino acids, which was less than that reported for the *B. stearothermophilus* enzyme [15]. The C-terminal amino acid sequence of the *B. stearothermophilus* enzyme was Ala-Ser-Glu-Phe-Leu-Gln-Asn-Gly-His-His-Ile-Leu-Ser-Arg-Arg-Arg-Ala-Arg. The amino acid sequence of the *B. stearothermophilus* enzyme obtained in this study is shown in Fig. 4. Structural genes for phosphotransbutyrilase and butyrate kinase were present at upstream and downstream of the *leudh* gene, respectively, and these genes were in an operon. This fact suggests that the *B. stearothermophilus* enzyme functions in leucine degradation.

3.5. Comparison of the amino acid sequence deduced from the leudh gene with the amino acid sequence obtained from X-ray analysis of the B. sphaericus enzyme

About 16% amino acid residues were different in the amino acid sequences deduced from the enzyme gene and obtained from X-ray analysis (Fig. 5). The majority of the X-ray sequence was determined from a combination of inspection of the electron density map and a consensus sequence from the *B. stearothermophilus* [15] and *T. intermedius* [11] leucine dehydrogenases. For a number of surface residues, the electron density was necessarily indistinct due to the movement of the side chains, and, in other places, the electron density was poor. It was also impossible to distinguish between aspartate and asparagine or glutamate and glutamine at the resolution of the map. Thus, the amino acid sequence deduced from the nucleotide sequence seems to be correct. However, there is a possibility that some amino acid residues may differ in the enzymes from *B. sphaericus* IFO 3525 and ATCC 4525, since Baker et al. [17] used the enzyme from *B. sphaericus* ATCC 4525.

3.6. Role of the C-terminal region of the enzyme

X-ray analysis of the enzyme suggested that the C-terminal regions of subunits interacted with each other (Fig. 6). To examine the role of the C-terminal region of the enzyme, the seven mutant enzymes, in which amino acid residues were deleted from the C-terminal of the enzyme, were obtained as described in the Section 2. The deleted mutant enzymes are designated LEUDEL-1, LEUDEL-2, LEUDEL-4, LEUDEL-6, LEUDEL-7, LEUDEL-9, and LEUDEL-14, in which 1, 2, 4, 6, 7, 9, and 14 amino acid residues are respectively deleted

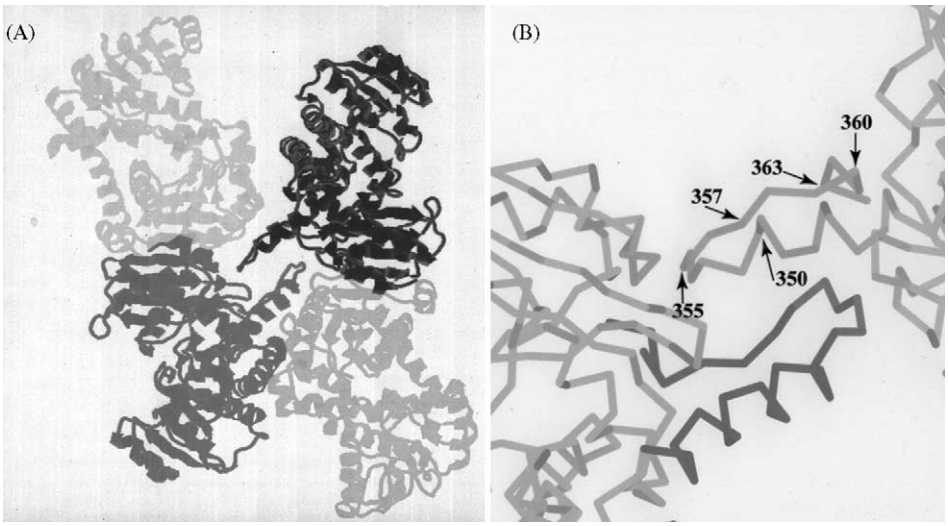

Fig. 6. Subunit interaction of leucine dehydrogenase from *B. sphaericus* (A) and the C-terminal regions in the subunit interaction (B). Numbers show the number of amino acid residues of the enzyme.

from the C-terminal of the enzyme. LEUDEL-1 and LEUDEL-2 maintained the same level of enzyme activity compared with the native enzyme. LEUDEL-4 showed weak enzyme activity (4.5% of the activity of the native enzyme). LEUDEL-6, LEUDEL-7, LEUDEL-9, and LEUDEL-14 did not show any enzyme activity at all. LEUDEL-1, LEUDEL-2, and LEUDEL-4 were purified to homogeneity, and their properties were compared with those of the native enzyme. LEUDEL-1 and LEUDEL-2 were octamers and showed the same V_{max} and K_m values as those of the native enzyme. However, LEUDEL-4 was a dimer and was different from the native enzyme in properties such as optimum pH, pH stability, thermal stability, and K_m values for NAD^+ and leucine (Table 1), though the substrate specificity for the oxidative deamination was similar to that of the native enzyme. These data suggest that the dimeric enzyme could not form the proper conformation in catalysis as that in the octameric enzyme. Thus, the C-terminal region, especially Asn-Ile-Leu-Asn residues, plays an important role in the interaction of the subunits and the quaternary structure formation of the enzyme.

The enzyme-overproducing *E. coli* cloned cells have potential for the effective production of branched-chain L-amino acids.

Table 1
Comparison of the properties of LEUDEL-4 with those of the native enzyme

Enzymes	Native enzyme	LEUDEL-4
Molecular mass (kDa)	340	82
Number of subunits	8	2
Optimum pH	11.0	10.0
pH stability	9.5–10.5	9.5
Thermal stability (°C)	55	40
K_m (mM)		
NAD^+	0.48	1.25
L-Leucine	3.3	11.0
L-Valine	3.8	25.0

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