

Enzyme with LeuDH activity, nucleotide sequence coding therefor and process for the preparation of the enzyme

- 5 The invention relates to an enzyme with LeuDH activity, to the *B. cereus* nucleotide sequence coding therefor, to microorganisms of the genus *E. coli* which contain said sequence, and to a process for the preparation of the enzyme.

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Leucine dehydrogenase (LeuDH, EC 1.4.1.9) is an NAD⁺-dependent oxidoreductase which catalyzes the reversible deamination of L-leucine and various other aliphatic L-amino acids to their keto analogues, the equilibrium of the

- 15 reaction lying on the L-leucine side.

The enzyme is suitable for the production of L-tert.-leucine and other non-proteinogenous aliphatic amino acids in the enzyme membrane reactor. L-tert.-Leucine functions as a chiral inductor in chemical synthesis and is used in

- 20 the preparation of drugs, bringing about an improvement in the metabolic stability of peptides and pseudopeptides.

The process for the preparation of L-leucine in a membrane reactor using LeuDH obtained from *B. stearothermophilus* has

- 25 been described by T. Oshima et al. (1).

At 72°C, however, the temperature optimum of LeuDH from *B. stearothermophilus* is more than 10°C higher than that of LeuDH from *B. cereus*.

- 30 This species has also been screened as an efficient enzyme producer.

Schütte et al. (2) have described the preparation and purification of the enzyme under discussion.

It has a molecular weight of ca. 310 kDa and consists of

- 35 eight identical subunits of 39 kDa each (3), in contrast to the other known types of LeuDH (the others have 6).

LeuDH from *B. cereus* has a temperature optimum of ca. 60°C and is stable up to 50°C in the pH range between 5.6 and 9.8 (heat for 30 min in 50 mM potassium phosphate buffer, pH

- 40 7.8, containing 0.1% 2-mercaptoethanol).

It can be stored in 50% glycerol at -20°C for at least one year without activity loss (2).

These properties make the increased use of LeuDH from *B. cereus* appear advantageous.

- 5 However, because of toxin formation, *B. cereus* is classified in hazard category 2 (German Law on Epidemics) and may only be handled if increased safety precautions are taken. This makes larger-scale enzyme production from the wild strain unattractive.

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The object of the invention is to provide the enzyme LeuDH known from *B. cereus* by another route.

- The invention provides a *B. cereus* DNA sequence (DNA fragment) which is characterized in that it codes for an enzyme with leucine dehydrogenase (LeuDH) activity and
- 15 comprises

- (a) the nucleotide sequence shown in Fig. 1 (sequence listing), or
- (b) a nucleotide sequence corresponding to the sequence of
- 20 (a) within the degeneracy of the genetic code, or
- (c) a nucleotide sequence hybridizing with the sequences of (a) and/or (b).

- The mutant of the DNA sequence (a) formed by single or
- 25 multiple base substitution, removal, insertion or exchange codes for an enzyme with identical biological activity to that of the DNA sequence (a).

- Fig. 1 shows the corresponding amino acid sequence between
- 30 codons 450 and 1550.

The DNA fragment (a) has an overall length of ca. 1.7 kb.

- The chromosomal DNA is isolated from the LeuDH-forming microorganism in a manner known per se. This is effected
- 35 for example by the process of H. Saito and K. Minra, Biochem. Biophys. Acta 72, 619 (1963), or Heinrichs et al. (4).

The resulting DNA is then cleaved in a manner known per se for ligation with plasmid DNA.

It is preferable to use restriction endonucleases which do not cut the LeuDH gene.

The ligation with the plasmid DNA is facilitated by choosing a restriction endonuclease (or restriction
5 endonucleases) for which there is only one cleavage site. The *B. cereus* DNA fragment according to the invention can be inserted by ligation into any plasmids capable of autonomous replication in *E. coli*.
Examples which may preferably be mentioned are ColE1,
10 pSC101, pBR322, pACYC177, pCR1 and especially pUC18.

The chromosomal DNA is ligated with the plasmid DNA in a manner known per se, for example by cleavage of the
15 chromosomal donor DNA and the plasmid with the same restriction endonuclease(s) and subsequent ligation of the cleavage products with a DNA ligase (11).

It is preferable to choose an SmaI cleavage site and an XbaI cleavage site.

20 With the commercially available pUC18 and the DNA fragment according to the invention, this gives pLeu2 of 4401 bp. This vector has been deposited in the *E. coli* strain XL1 blue under the number DSM 10441 in the German Collection of Microorganisms and Cell Cultures in accordance with the
25 Budapest Convention.

Fig. 2b shows the corresponding restriction map. Vector pLeu2 can undergo autonomous replication in *E. coli*.

30 The vectors carrying the DNA fragment according to the invention can be transformed in a manner known per se to microorganisms of the genus *E. coli*.

The enzyme with LeuDH activity is constitutively strongly overexpressed in *E. coli* under the endogenous LeuDH
35 promoter from *B. cereus* and is present in the *E. coli* cells in a proportion of up to ca. 50% of the soluble cell protein.

This value exceeds the value found for expression in *B. cereus* by a factor of 10 to 30.

The invention further relates to the use of the vectors containing the DNA fragment according to the invention, in the appropriate transformants, for the preparation of enzymes with LeuDH activity.

- 5 The media to be used for incubation of the transformants are synthetic, semisynthetic or natural media such as those in general use.

- 10 They contain nutrients like carbon sources, nitrogen sources and inorganic compounds.

Examples of favourable carbon sources are sugars such as glucose, fructose or invert sugar, saccharified starch, sorbitol or glycerol.

- 15 Examples of favourable nitrogen sources are ammonium sulphate, ammonium chloride, ammonium nitrate, ammonium phosphate, ammonium hydroxide, ammonium tartrate, ammonium acetate or urea.

- 20 Examples of favourable nutrients which can be offered as both nitrogen and carbon sources are peptone, meat extract or corn steep liquor.

- Examples of favourable inorganic compounds are potassium dihydrogen phosphate, dipotassium hydrogen phosphate, sodium hydrogen phosphate, disodium hydrogen phosphate, 25 magnesium sulphate, magnesium chloride, potassium chloride, sodium chloride, iron(II) sulphate, iron(II) chloride, iron(III) sulphate, iron(III) chloride, manganese sulphate or manganese chloride.

- 30 Examples of other favourable nutrients are yeast extract or vitamins.

- The transformants can be incubated either in a liquid medium or in a solid medium. It is generally advantageous to use a liquid medium. For large-scale production it is particularly advantageous to carry out the incubation with 35 shaking or with aeration and stirring. The incubation temperature is 20 to 45°C, preferably 28 to 37°C. It is advantageous to adjust the medium to a pH of 6 to 9 with a suitable neutralizing agent during the incubation. As a rule the enzyme is present inside the cells.

After expression of the recombinant LeuDH DNA in the chosen *E. coli* strain, the cells are therefore macerated (e.g. by grinding with glass beads) and the desired enzyme is obtained from the resulting crude extract by methods which are generally known.

This crude extract can also be used for the activity test. In this way it is found, in one Example, that the specific LeuDH activity of the enzyme obtained from the recombinant clone according to the invention has increased by a factor of ca. 27 compared with the crude extract similarly obtained from *B. cereus* (0.6 u/mg → 16 u/mg).

The invention also provides the enzyme obtained in this way and its use for the enzymatic synthesis of non-proteinogenous, especially aliphatic L-amino acids by reductive amination of the corresponding α -keto acids.

L-tert.-Leucine is preferably used.

Examples of starting compounds used for the corresponding L-amino acids are 2-oxo-4-methylpentanoic acid (ketoleucine), 2-oxo-3-methylpentanoic acid (ketoisoleucine), 2-oxo-3-methylbutanoic acid (ketovaline), 2-oxopentanoic acid (ketonorvaline), 2-oxo-4-methylhexanoic acid, 2-oxobutanoic acid, 2-oxo-3,3-dimethylbutanoic acid (keto-tert.-leucine), 2-oxohexanoic acid (ketonorleucine), 2-oxo-4,4-dimethylpentanoic acid (ketoneopentylglycine), 2-oxo-3,3-dimethylpentanoic acid, 2-oxo-4-ethylhexanoic acid, 2-oxo-5,5-dimethylhexanoic acid, 2-oxo-3-cyclohexylpropanoic acid and 2-oxo-4,4-dimethylhexanoic acid.

Characteristic features of the enzyme obtained according to the invention are an increased initial activity and a lower product inhibition compared with enzymes known for these purposes from the state of the art.

Experimental section

1. Isolation of genomic DNA from *B. cereus*

- 5 *B. cereus* DSM 626 was inoculated from the strain collection as an overnight culture in 4 ml of 2% yeast extract, 0.2% K_2HPO_4 and 4% glucose and shaken at 30°C. The preculture was used to inoculate 200 ml of the same medium (1:40 dilution) and the cells were left to grow to an OD_{600} of
- 10 1.43 (4 h at 30°C). 1 g of the resulting 2 g of bacterial cells was used for isolation of the genomic DNA. The DNA was isolated essentially by the method of Heinrichs et al. (4). For efficient removal of the DNA, RNase A (0.1 mg/ml) was added as well as the protease (Quiagen). The yield
- 15 from 1 g of cells (moist weight) was 1.8 mg of genomic DNA.

2. Preparation of a PCR fragment of LeuD_H

- A genome bank was prepared by synthesizing two degenerate
- 20 primers
 (TTCGAA/GTATT/CTA/G/TGAAAAATAT/CGATTATGAG/ACAA
 GGCA/GTTGATCACATAATCCGGG/CGCATAGACGAT),
 which were derived from *B. cereus* protein sequences and the known sequences of already cloned LeuD_Hs from *T.*
- 25 *intermedius* (5) and *B. stearothermophilus* (6).
 The codon usage of *B. cereus* was taken from a Table which is to be found on the Internet under

[http://www.dna.affrc.go.jp/nakamura-bin/showcodon.cgi?species=Bacillus + cereus + \[gbbct\]](http://www.dna.affrc.go.jp/nakamura-bin/showcodon.cgi?species=Bacillus+cereus+[gbbct])

Codon usage table

Bacillus cereus [gb|ccr]: 37 CDS's (10419 codons)

fields: [triplet] [frequency, per thousand] ([number])

UUU 25.2(263)	UCU 19.8(206)	UUA 32.0(333)	UGU 3.3(34)
UUC 13.5(141)	UCC 2.1(22)	UAC 10.5(109)	UGC 1.8(19)
UUA 43.1(449)	UCA 18.2(190)	UAA 2.6(27)	UGA 0.1(1)
UUG 8.2(85)	UCG 2.8(29)	UAG 0.9(9)	UGG 15.8(163)
CUU 14.0(146)	CCU 10.6(110)	CAU 16.1(168)	CGU 14.3(149)
CUC 1.7(18)	CCC 0.6(6)	CAC 4.2(44)	CGC 4.7(49)
CUA 10.3(107)	CCA 17.9(186)	CAA 27.2(283)	CGA 4.7(49)
CUG 2.9(30)	CCG 4.3(47)	CAG 7.6(79)	CGG 0.6(6)
AUU 41.6(433)	AUC 15.8(163)	AUA 42.8(446)	AGU 13.6(142)
AUG 10.6(110)	AAC 1.9(20)	AAC 14.7(155)	AGC 7.0(73)
AUA 13.1(136)	AAA 31.3(326)	AAA 60.3(628)	AGA 5.9(61)
AUG 22.7(237)	ACG 13.9(145)	AAG 16.9(176)	AGG 2.3(24)
GUU 24.1(251)	GCU 25.9(270)	GAU 48.2(502)	GGU 26.7(278)
GUC 3.0(31)	GCC 3.3(34)	GAC 9.7(101)	GGC 6.9(72)
GUA 33.0(344)	GCA 35.8(373)	GAA 46.0(479)	GGA 31.1(324)
GUG 9.4(98)	GCG 12.2(127)	GAG 15.2(158)	GGG 9.7(102)

Coding GC 36.42% 1st letter GC 48.18% 2nd letter GC 36.50% 3rd letter GC 24.57%

Codon usage for each CDS (format)

With these

primers and the genomic DNA as template, an 850 bp DNA fragment could be synthesized in the PCR (100 ng of template, 40 pmol of each primer, 200 μ mol of dNTPs, 1.5 mM $MgCl_2$, 2.5 u of Taq polymerase, annealing temperature 42°C, 35 cycles: 1.: denaturation: 5 min at 94°C, 2.-34.: denaturation: 1.30 min at 94°C, annealing: 1.30 min at 42°C, synthesis: 72°C, 35.: last synthesis: 7 min at 72°C). The DNA fragment was cloned into vector pUC18 and sequenced according to (7).

3. Detection of the LeuD^H gene by Southern blotting

To prepare a DIG-labelled DNA probe, the PCR was repeated with the cloned LeuD^H fragment as template and the above
5 primers, using DIG-labelled dUTPs (66 μ M DIG-11-dUTP, 66 μ M dTTP, 134 μ M dATP, dCTP, dGTP) (8).

The genomic DNA was cleaved with various restriction
enzymes and separated by electrophoresis on a 0.8% agarose
10 gel (50 V, 5 h, RT) and the separated DNA was transferred to a nylon filter and, after UV crosslinking, hybridized with the DNA probe (8). The bound DNA was visualized by chemiluminescence (reagent: CSPD, Luminograph) or by means of a colour reaction (8). The DNA cleaved with the enzyme
15 XbaI showed a band of ca. 3000 bp after hybridization, which was suitable for creating a partial gene bank.

4. Creation of a partial gene bank and screening with an activity test

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The genomic DNA separated in the 2000-3000 bp region on the agarose gel was cut out of the gel with the agarose and isolated from the gel with glass milk (Jetsorb). The DNA purified in this way was cloned into vector pUC18 and
25 transformed in *Escherichia coli* XL1 blue (9). Ca. 500 colonies were picked from this partial genome bank and transferred to master plates.

The colonies were transferred to a Whatman filter and subjected to an activity test (6, 10). A clone giving an
30 intense blue colouration was found.

5. Characterization of the recombinant *E. coli* strain

5.1. Determination of the DNA sequence

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The plasmid DNA was isolated from the positive *E. coli* clone and a gene map was created by digestion with various restriction enzymes. The plasmid was called pLeu1 (see plasmid map, Fig. 2a).

Plasmid pLeu1 was used to prepare plasmid pLeu2 (Fig. 2b), in which the non-translated region at the 5' end of the gene was shortened to 400 bp by digestion with the restriction enzyme SmaI. Subgenomic fragments were cloned from this plasmid and the complete DNA sequence was determined using the universal and reversal primers from Pharmacia and 5 sequence-specific primers (7). Both strands were sequenced (Fig. 1). The strain *E. coli* XL1 blue pLeu2 was deposited under no. DSM 10441 in the German Collection of Microorganisms and Cell Cultures in accordance with the Budapest Convention.

5.2. Expression of the recombinant LeuDH DNA in *Escherichia coli*

5.2.1. Activity test

The recombinant clone was grown overnight in 5 ml of LB medium and the bacteria were centrifuged off, resuspended in 1.6 ml of PBS and then macerated with glass beads (0.3 mm diameter) (2 min in a Vortex). The resulting crude extract was used in an activity test to show an activity of 16 u/mg. The LeuDH activity of the recombinant clone has accordingly been increased by a factor of 30 compared with the crude extract from *B. cereus* (0.6 u/mg) (2).

5.2.2. Test procedure

3 ml scale

Solutions required: 0.1 M glycine in 0.1 M NaCl solution
 0.1 M NaOH
 52.2% of sol. a + 47.8% of sol. b gives
 buffer solution of pH ca. 10.7.
 13.53 mg/ml of KPI, pH 8.0, NAD (20.4 mM)
 8.59 mg/ml of KPI, pH 8.0, L-leucine (65.5 mM)

Assay: 2000 µl of 0.1 M glycine/KCl/KOH buffer,
pH 10.7
500 µl of NAD
500 µl of L-leucine
5 25 µl of sample

Temp.: 25°C, λ = 340 nm, factor: 19.453

5.3.3. Preparation of LeuDH

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The strains were fermented in the following media:

B. cereus (DSM 626): 2.0% (w/v) of yeast extract
0.2% (w/v) of K_2HPO_4
15 4.0% (w/v) of glucose
pH 7.0
highest possible oxygenation during the
fermentation
temp.: 30°C

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The growing time at 1% inoculation volume is ca. 6 to 8 h;
expected OD_{562} ca. 50; harvest at 1% residual glucose.

E. coli pLeu2: LB medium
25 0.5% (w/v) of NaCl
0.5% (w/v) of yeast extract
1.0% (w/v) of bactotryptone
100 mg/µl of ampicillin
pH 7.0 to 8.0
30 pO_2 : 70 to 90%
temp.: 37°C

Growing time at 5% inoculation volume ca. 5 h; harvest at
 OD_{550} = 3.3.

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The following values are obtained:

Strain	Fermenta tion volume (l)	Moist weight of bacteria (kg)	Total activity (u)	Volume activity (u/l)	Activity / moist weight (u/kg)
B. cereus	200	70.7	143,000	715	2023
E. coli pLeu2	20	0.075	8250	412	110,000

Literature

1. Oshima et al., Biotechn. Bioeng. Vol XXVII (1985) 1616-1618
- 5 2. Schütte et al., Appl. Microbiol. Biotechnol., 22, 306-317
3. Lünsdorf et al., FEBS Lett., 193(2) (1985) 261-265
4. Heinrichs et al., J. Bacteriol., 175 (1993) 6760-6766
5. Oshima et al., Eur. J. Biochem., 222, (1994), 305-312
- 10 6. Nagata et al., Biochemistry, 27 (1988) 9056-9062
7. Sanger et al., Proc. Nat. Acad. Sci. USA, 74 (1977) 5463-5467
8. Boehringer Mannheim, data sheet on "DNA labeling and detection kit: nonradioactive."
- 15 9. Bullock et al., BioTechniques, 5 (1987) 376-378
10. Raetz, C.R.H., Proc. Nat. Acad. Sci. USA, 72 (1975) 2274-2278
11. Sambrook, J., Fritsch E.F. and Maniatis, T. (1989) Molecular Cloning. A Laboratory Manual 2nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
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Claims

1. A *B. cereus* DNA sequence, characterized in that it codes for an enzyme with leucine dehydrogenase (LeuDH) activity and comprises
 - (a) the nucleotide sequence shown in Fig. 1 (sequence listing), or
 - (b) a nucleotide sequence corresponding to the sequence of (a) within the degeneracy of the genetic code, or
 - 10 (c) a nucleotide sequence hybridizing with the sequences of (a) and/or (b), Fig. 1 forming part of this Claim.
2. A vector capable of autonomous replication in *E. coli*, into which the nucleotide sequence according to Claim 1 is
15 inserted.
3. A vector according to Claim 2, characterized in that it contains DNA from plasmid pUC18 and the DNA sequence according to Claim 1, deposited as pLeu2 in the strain *E. coli* XL1 blue under no. DSM 10441 and characterized by the
20 restriction map of Fig. 2b, said Figure forming part of this Claim.
4. A microorganism of the genus *E. coli* with a high
25 productivity for LeuDH, containing a vector according to Claims 2 and 3.
5. A microorganism of the genus *E. coli* according to Claim 4, deposited as the strain *E. coli* XL1 blue under no.
30 DSM 10441.
6. A process for the preparation of leucine dehydrogenase (LeuDH), characterized in that recombinant DNA containing
 - a) the nucleotide sequence derived from *B. cereus* according to Claim 1 and
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 - b) plasmid DNA which undergoes autonomous replication in *E. coli* is inserted in the form of a vector into a microorganism of the genus *E. coli*, the resulting transformant is fermented in a suitable medium and the
40 Leu-DH is separated from the microorganism.



7. . A process according to Claim 6, characterized in that a microorganism according to Claim 4 or 5 is used.
- 5 8. . Use of the enzyme with LeuDH activity obtained according to Claims 6 and 7 for the preparation of non-proteinogenous L-amino acids from the corresponding α -keto acids.
- 10 9. . Use of the enzyme according to Claims 6 and 8, characterized in that the following α -keto acids are used: 2-oxo-4-methylpentanoic acid, 2-oxo-3-methylpentanoic acid, 2-oxo-3-methylbutanoic acid, 2-oxopentanoic acid, 2-oxo-4-methylhexanoic acid, 2-oxobutanoic acid, 2-oxo-3,3-
- 15 dimethylbutanoic acid, 2-oxohexanoic acid, 2-oxo-4,4-dimethylpentanoic acid, 2-oxo-3,3-dimethylpentanoic acid, 2-oxo-4-ethylhexanoic acid, 2-oxo-5,5-dimethylhexanoic acid, 2-oxo-3-cyclohexylpropanoic acid and 2-oxo-4,4-dimethylhexanoic acid.