Appl Microbiol Biotechnol (1985) 22:306–317

Applied and Microbiology Biotechnology

L-Leucine dehydrogenase from *Bacillus cereus*

Production, large-scale purification and protein characterization

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Summary. An improved method for the production of L-leucine dehydrogenase is described employing a mutant with a constitutive enzyme and a fed-batch cultivation technique yielding high cell concentrations. Purification of L-leucine dehydrogenase to homogeneity was carried out starting with 30 kg *Bacillus cereus* cells by heat treatment at 63 °C, followed by two liquid-liquid extraction steps and three conventional column chromatographies. Crystals have been obtained from the 95-fold purified enzyme. The molecular weight of the native enzyme was determined by sedimentation equilibrium and gel filtration studies to be 310000 containing eight identical subunits with a molecular weight of 39000. The sedimentation coefficient was estimated to 11.65 S. Branched-chain amino acids like L-leucine, L-valine or L-isoleucine are deaminated by the NAD-dependent enzyme. In the reverse reaction a variety of 2-ketoacids, especially 2-ketoisocaproate, 2-keto-isovalerate and 2-keto-3-methyl-valerate, were reductive aminated to the corresponding L-amino acids in the presence of 0.9 M ammonia. The amino acid composition for the subunit of L-leucine dehydrogenase is presented.

Introduction

L-Leucine dehydrogenase from *Bacillus cereus* was first isolated and partially purified by Sanwal and Zink (1961). The enzyme catalyzes a reversible reaction, the oxidative deamination of L-leucine and reductive amination of the corresponding keto acid. The equilibrium of the reaction favours the synthesis of L-leucine. To demonstrate that the oxidative deamination of various branched- and straight-chain amino acids is due to the broader substrate specificity of L-leucine dehydrogenase rather than the nonspecific action of glutamate or alanine dehydrogenase, Zink and Sanwal (1962) also purified a L-leucine dehydrogenase from a mutant of *Bacillus subtilis* which is defective in alanine as well as glutamate dehydrogenase activities.

L-leucine dehydrogenase is present in both vegetative cells and spores of various *Bacillus* species. The crystallization and enzymological characterization of the enzyme from *Bacillus sphaericus* have been reported (Soda et al. 1971; Ohshima et al. 1978). We described previously improved conditions for production and an efficient method for the large-scale purification of the enzyme from *B. sphaericus* (Hummel et al. 1981).

In this paper we show that the cellular level of L-LeuDH in a constitutive mutant of *B. cereus* remains high even at the cell density up to 20% (w/v). The introduction of liquid-liquid extraction techniques allows the purification of the enzyme at room temperature in high yield and short time. Besides native NADH, the enzyme can also utilize the molecular weight enlarged PEG 20000-NAD(H) derivative as the coenzyme and has been applied for the continuous production of L-amino acids in an enzyme membrane reactor as described by Wandrey et al. (1984a). Properties which are important for application of this enzyme as a technical catalyst are studied in detail.

Materials and methods

Microorganisms. Bacillus sphaericus (DSM 396) and *Bacillus cereus* (DSM 626) were obtained from the German Collection of Microorganisms (DSM, Göttingen, W. Germany).

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Materials

The L-amino acids and the 2-keto acids were obtained from Sigma (München, W. Germany) or Roth (Karlsruhe, W. Germany), respectively. The coenzymes were products from Boehringer (Mannheim, W. Germany). PEG 1540 (pure grade) was purchased from BP-Chemie (Düsseldorf, W. Germany). Potassium phosphate, sodium chloride and ammonium sulfate are products from Merck (Darmstadt, W. Germany). DEAE-Sephacell, Sepharose 4B and Sephacryl S-200, Superose 12 and S-400 Superfine for chromatography were obtained from Deutsche Pharmacia (Freiburg, W. Germany), Romicon Hollow Fiber Cartridge type HF 30-20-GM 80 and Amicon Hollow Fiber Cartridge type XM 50 from Amicon GmbH (Witten, W. Germany). Yeast extract was purchased from Ohly GmbH (Hamburg, W. Germany), glucose for large-scale fermentation from Maizena (Krefeld, W. Germany), other components for the cultivation of microorganisms were products from Merck (Darmstadt, W. Germany).

Methods

Cultivation of *Bacillus cereus* in a 600 l batch culture: *Bacillus cereus* (DSM 626, wild type) cultivated by a 600 l batch cultivation in a medium containing 4% glucose, 1% yeast extract, 0.2% K_2HPO_4, pH 7.0, served as starting material for large-scale enzyme purification. During growth the pH was held constant between 6.8 and 7.2 by adding 3 *N* KOH or 3 *N* H_3PO_4, respectively. The temperature was maintained at 30° C and the aeration rate set to 1.5 vvm. Cells were harvested after 20 h of growth, yielding 30 kg of wet cell mass (50 g per l) containing 434,000 units of LeuDH with a specific activity of 0.6 U/mg.

Media for isolation of mutants possessing a constitutive LeuDH: *Medium I* (containing per 1 ltr):

4.8 g $K_2HPO_4 \cdot 3\,H_2O$
1.5 g KH_2PO_4
0.5 g Na-citrat $\cdot 2\,H_2O$
0.2 g $MgSO_4 \cdot 7\,H_2O$
2 mg $CaCl_2 \cdot 2\,H_2O$
0.4 mg $ZnSO_4 \cdot 7\,H_2O$
0.2 mg $FeCl_3 \cdot 6\,H_2O$
5 g Glucose
1.1 g L-leucine

Medium II: According to medium I, but 1.0 g $(NH_4)_2SO_4$ instead of leucine.

Enrichment and isolation of mutants constitutive for L-leucine dehydrogenase (Obermeier and Poralla 1976): *Bacillus cereus* (DSM 626) was cultivated in 500 ml erlenmeyer flasks filled with 100 ml medium. After growth for 24 h 5 ml culture broth are transferred into fresh medium I. This transfer is repeated four more times. After that an aliquot is plated on medium I and incubated for 2 days. Larger colonies are selected and purified to yield single colonies. To find a mutant with a constitutive L-leucine dehydrogenase activity each isolate was cultivated in a glucose-ammonia medium (medium II) and the specific activity of the enzyme determined.

Fed-batch cultivation

In a 10 l-bioreactor, cultivation is started with 8 l of a medium containing: 4% yeast extract; 2% glucose; 0.2% KH_2PO_4; 5 ml antifoam (Ucolup N 115, Brenntag, Hamburg, W. Germany) with a final pH of 7.2. The bioreactor was inoculated with 250 ml of a culture grown for 24 h at 30° C and 120 rpm on a reciprocal shaker. Growth in the bioreactor proceeded at 30° C with an aeration rate of 1.5 vvm using a flat blade turbine stirrer at 250 rpm. Consumption of glucose causes a decrease of pH to about 5.5 during the first five hours of growth. Later on the pH begins to increase again (degradation of amino acids); when it reached 6.5 addition of a 50% solution of glucose started. The fed substrate is added every 2 min in a short time impulse (2 s, yielding about 20 ml). After approximately ten minutes the pH begins to fall again and feeding is stopped at pH 6.3. During one cycle 60—80 ml feed glucose are added; the cycle repeats itself after about 30 min.

Analytical methods

Enzymatic activity was measured at 340 nm and at 30° C using a Beckman DU5 photometer. The standard assay mixture for the oxidative deamination reaction contained in 100 mM glycine/NaCl/NaOH buffer (pH 10.7), 10 mM L-leucine and 3.4 mM NAD^+ and for the reductive amination reaction in 900 mM NH_4Cl/NH_4OH buffer (pH 9.5), 4.5 mM sodium 2-ketoisocaproate and 0.204 mM NADH. Both reactions were performed in a final volume of 3 ml and initiated by the addition of limiting amounts of enzyme. One unit of L-leucine dehydrogenase was defined as the amount of enzyme which catalyzes the production (or consumption) of 1 µmol of NADH per min under the standard assay conditions. Specific activity is expressed as units per milligram of protein. Protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin for calibration.

The concentration of purified enzyme was measured by absorbance at 280 nm using a conversion value of $E^{1\%}_{1\,cm} = 7.98$ which conforms to the protein content determined by the Bradford method.

Gel electrophoresis

Disc gel electrophoresis was carried out according to Jovin et al. (1964) at room temperature in a Tris/glycine buffer. The gels were stained with Coomassie brilliant blue. To ascertain that the stained band after disc gel electrophoresis corresponds with the L-leucine dehydrogenase some gels were cut consecutively into slices of about 2.5 mm thickness from which the protein was eluted with buffer and the enzyme activity determined.

The molecular weight of the polypeptide chains was determined according to Shapiro et al. (1967) using hemoglobin, chymotrypsinogen, pepsin, ovalbumin and bovine serum albumin as marker proteins.

Molecular weight determination

The molecular weight of the homogeneous L-leucine dehydrogenase was estimated by gelfiltration on Sephacryl S-400 Superfine (1.6 × 92 cm) calibrated with appropriate molecular weight standards by the method of Andrews (1965). The column was equilibrated with 50 mM potassium phosphate buffer (pH 7.5) containing 0.15 M NaCl. Elution was carried out at a flow rate of 10 ml/h.

The molecular weight was also estimated by the use of high performance gel filtration using a Superose 12 column (HR 10/30) together with the Pharmacia Fast Protein Liquid

Chromatography (FPLC) system. 200 µl of the enzyme or marker protein was applied to the column equilibrated against the buffer described above. The flow rate was 0.4 ml/min and 0.2 ml fractions were collected. The enzyme was detected by UV-monitor and assay in the eluate.

The following marker were used for calibration for both types of gel filtration: Blue Dextran 2000, thyroglobuline (669,000), ferritin (440,000), catalase (232,000), aldolase (158,000) and bovine serum albumin (67,000).

Ultracentrifuge

Ultracentrifugation experiments were carried out in a Beckman Spinco model E analytical centrifuge. For the measurements an improved collimatic optic (Flossdorf 1980) was used. The instrument was equipped with RTIC temperature control unit, monochromator and photoelectric scanner. The homogeneous enzyme was dialyzed against 20 mM potassium phosphate buffer, pH 7.5. Centrifugation was carried out in a four-place rotor AN-F using Kel-F double sector cells (12 mm) with sapphire windows. The wavelength for all measurements was 280 nm.

Spectrophotometric studies

The UV and visible spectra of the purified enzyme were measured in a Perkin Elmer recording spectrophotometer (Model 554). Aliquots of the enzyme stock (in 50% glycerol) were taken and diluted to appropriate concentration with water, followed by dialysis against 0.01 M potassium phosphate buffer, pH 7.0. Before measurement, samples were clarified by high-speed centrifugation.

For determination of the tryptophan and tyrosine contents of the enzyme, the protein samples were prepared by dialysis against 6 M guanidinium hydrochloride at pH 12.5 according to Edelhoch (1967). Spectrophotometrical titration of the free sulfhydryl groups of the enzyme using 5,5'-dithiobis(2-nitrobenzoic acid) was carried out in 0.1 M potassium phosphate buffer, pH 7, containing 8 M urea and 5 mM EDTA at room temperature. The release of nitromercapto benzoate anion was monitored by absorbance at 412 nm (Habeeb 1972).

Amino acid composition and peptide mapping

Amino acid composition of the enzyme was determined using an automatic amino acid analyzer, Biotronik LC 6000 (München, W. Germany), equipped with an integrator. Samples were hydrolyzed with 6 N HCl at 105° C for 24 to 72 h. For determination of the total content of half-cystine, protein samples were oxidized with performic acid (Hirs 1967) prior to acid hydrolysis. Tryptic digest and peptide mapping were performed as previously described (Tsai et al. 1974).

Results

Selection of mutants of Bacillus cereus with a constitutive leucine dehydrogenase

Leucine dehydrogenase activity in strains of *Bacillus* can be induced by isoleucine or valine (Poralla 1971). A constitutive mutant was desirable to avoid the addition of an inducer during the enzyme production. The search for such a mutant was focused on one strain of *B. sphaericus* (DSM 396) and one of *B. cereus* (DSM 626), which are both known to be good producers of LeuDH (Ohshima et al. 1971; Hummel et al. 1981). Preliminary growth experiments in a minimal medium (results not shown), however, revealed that only *B. cereus* is able to grow under these conditions. Therefore, we used this strain only for further experiments. In Table 1 some of the isolates from the enrichment procedure are listed which grow more abundantly on L-leucine than the wild type. When cultivated in a glucose-ammonia medium (medium II) almost all of these strains show higher enzyme activity than the parent strain. To find a suitable production strain, we tested some isolates under production conditions.

Table 1. Specific activity of leucine dehydrogenase in isolates of *Bacillus cereus*. Isolates are selected by their ability to give good growth on L-leucine. Enzyme activity was measured after growth in a glucose-ammonia medium

Isolate	L-leucine dehydrogenase (U/mg)
wild typ	0.03
IV/8	1.63
III/3	0.22
IV/5	0.21
IV/1	0.18
K 3	0.15
K 4	0.12
IV/3	0.02

Production of L-leucine dehydrogenase in a bioreactor

Culture conditions were investigated to increase the cell yield during cultivation. A fed-batch process with a feedback control based on pH made it possible to use high amounts of glucose which otherwise inhibit growth in batch culture. Under these conditions we gained cell suspensions with 170—200 g wet cell mass per 1 l.

Some of our isolated mutants containing a constitutive LeuDH were compared at 10 l scale in these fed-batch process. Figure 1a—c show the enzyme level of the wild type compared to isolates IV/8 and III/3. The wild type shows an enzyme content of 1,400 Units/l after 20 h. During further growth the enzyme level remains constant, and thus the specific enzyme activity decreases. During the cultivation of the isolate IV/8 the enzyme production remains correlated to the growth for

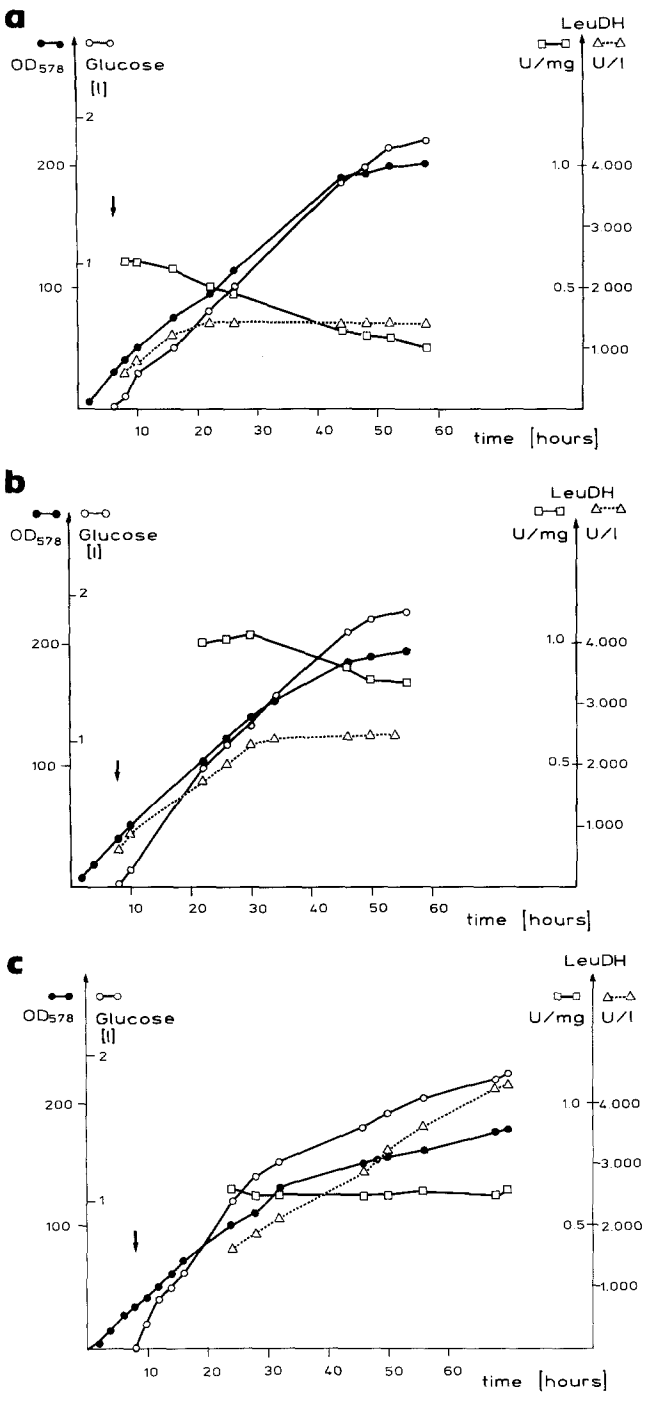


Fig. 1. Production of leucine dehydrogenase in a 10 l fed-batch cultivation of *Bacillus cereus*. **a** wild type; **b**, **c** mutants possessing a constitutive L-leucine dehydrogenase. ↓ = start of glucose feeding. b = strain IV/8; c = III/3

some time; however, in later stages the enzyme production is lowered. With this strain about 2,400 Units/l after 45 h can be obtained. Isolate III/3 produces LeuDH corresponding to the cell growth during the whole cultivation period of about 70 h. The enzyme content after that time is about 4,300 Units/l, while the specific enzyme activity remains constant over the whole period. Table 2 summarizes the data for production of leucine dehydrogenase with 3 strains of *B. cereus*.

Table 2. Enzyme production with different strains of *Bacillus cereus* (Data from experiments in a 10 l-scale)

	Wild type	IV/8	III/3
Wet cell mass (g/l)	135	120	110
Enzyme content (U/l)	1,400	2,400	4,200
Cellular enzyme content (U/kg wet cell mass)	10,400	20,000	38,200
Specific enzyme activity (U/mg)	0.25	0.81	0.65

Purification of L-leucine dehydrogenase

Thirty kilogram *Bacillus cereus* cells were desintegrated using a continuously operating industrial agitator mill (Netzsch LME 20) followed by a heat treatment for 10 min at 63 °C (Schütte et al. 1983). The pH-value selected for the heat denaturation procedure is very important and should be in the range of pH 7.5 to 8.5 for a good recovery of the enzyme (Fig. 2).

Removal of cell debris and coagulated proteins

Although insoluble material can be conventionally separated from the desired L-leucine dehydrogenase fraction after the heat treatment in a

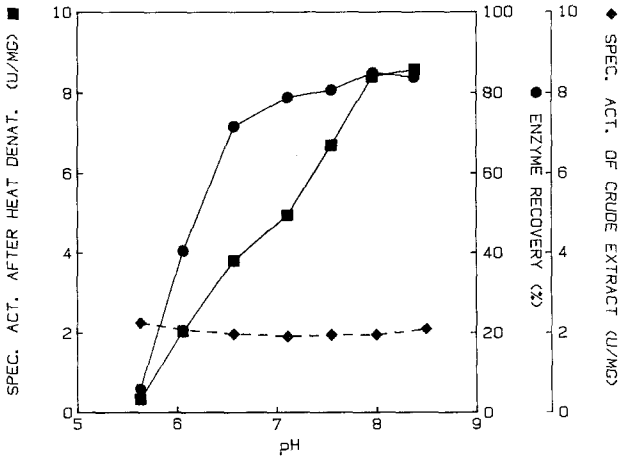


Fig. 2. Influence of pH on the heat denaturation of L-leucine dehydrogenase from *B. cereus*. Enzyme recovery ●—● and specific activity ■—■ were measured after 10 min heating time at 63 °C

tubular centrifuge (Hummel et al. 1981), we developed an extraction process in aqueous two-phase systems to shorten the operating time for this step, and at the same time to improve the specific activity of the enzyme.

For extraction of *B. cereus* homogenate polyethylene glycol 1540 and potassium phosphate salt (pH 8.0 mixture) were added and stirred for 1 h to form a PEG/salt system and ensure equilibrium of partition. The whole extraction system consisted of 18% (w/w) polyethylene glycol 1540, 7% (w/w) potassium phosphate salt, 70 l crude extract (final cell concentration 20% w/w before disruption) and deionised water to give a 140 kg (128 l) phase system. Liquid-liquid separation was carried out employing a disc stack separator (Westfalia separator model SAOH-205) with a feed rate of 100 l/h. 98% of the L-leucine dehydrogenase was found in the polyethylene glycol rich upper phase I (86 l) which is essentially free from any solid material. After this step the specific activity of the enzyme was increased 11 times in comparison to the crude extract, which is about 2.2 times higher than that reported for the conventional centrifugation step.

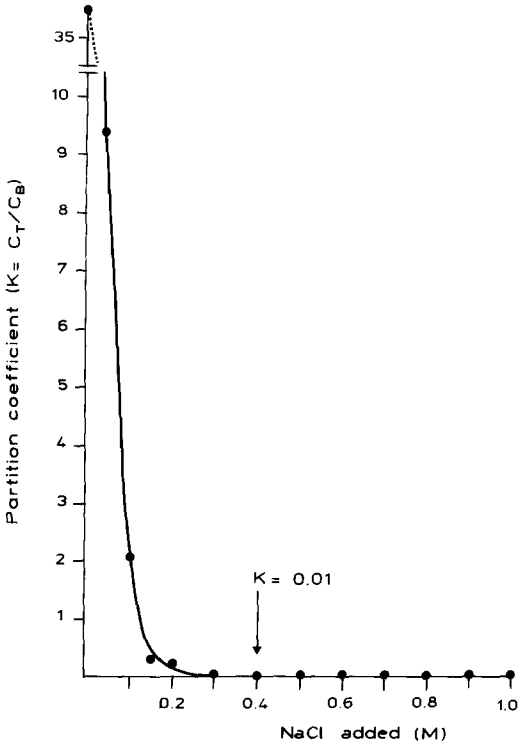


Fig. 3. Dependence of the partition coefficient of L-leucine dehydrogenase on the concentration of sodium chloride in a secondary phase system (50% upper phase I, 11% potassium phosphate, pH 7.0)

Further purification

By adding salt to top phase I, conditions could be established that induced the formation of two liquid phases and partitioned the enzyme this time into the salt-rich lower phase. The second phase system had a final volume of 172 l and contained 86 l of the upper phase I, 11% (w/v) potassium phosphate salt (pH 7.0 mixture) and sodium chloride to a final concentration of 0.3 M. After stirring for 1 h separation of the phases was allowed to proceed under gravity in a settling tank. Complete phase separation could be accomplished within 30 min. Figure 3 shows that the addition of sodium chloride is very important to shift the L-leucine dehydrogenase to the salt rich lower phase II. After separation the lower phase II was diafiltrated against 10 mM potassium phosphate buffer pH 7.5 and concentrated using a Romicon Hollow Fiber Cartridge (Type HF 30 — 20-GM 80). Final concentration to 2.4 l was accomplished using an Amicon Hollow Fiber Cartridge (Type XM 50, 2.5 FT^2). Then the enzyme solution was applied to a DEAE-Sephacell column (21 × 60 cm) equilibrated against dialysis buffer. After an extensive wash with starting buffer the protein was eluted by increasing the sodium chloride concentration gradually from 0 to 0.25 M in starting buffer. The L-leucine dehydrogenase eluted at the end of the 100 l gradient totally separated from the NADH-oxidase which is producing a blank during the enzyme assay. The active fractions were concentrated by ultrafiltration and gelfiltrated on a Sephacryl S-200 Superfine column (10 × 80 cm) at a flow rate of 88 ml/h.

Final purification to homogeneity was carried out using interfacial salting out chromatography on Sepharose 4B (von der Haar 1976). Therefore the enzyme solution, obtained by gelfiltration, was dialyzed against 50 mM potassium phosphate buffer, pH 7.5, containing ammonium sulfate at 35% saturation. The precipitate formed overnight was collected by centrifugation and discarded. The supernatant was applied to a Sepharose 4B column (5 × 32 cm) equilibrated against the dialysis buffer. Unbound protein was washed off with starting buffer. L-leucine dehydrogenase was eluted by applying a linear decreasing 6 l-gradient from 40% to 20% ammonium sulfate. The enzyme appeared in the effluent at an ammonium sulfate saturation of 35—30% as can be seen in Fig. 4. Homogeneous L-leucine dehydrogenase fractions were collected, concentrated by ultrafiltration and dialyzed against 50 mM potassium phosphate

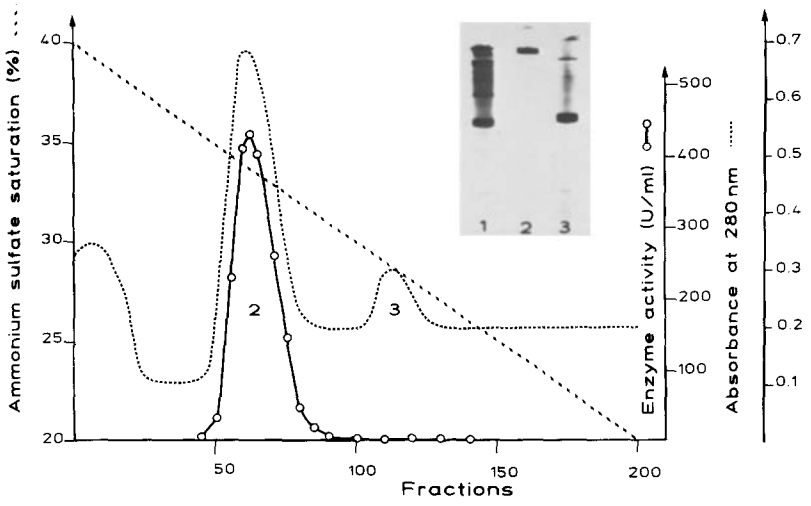


Fig. 4. Elution profile of L-leucine dehydrogenase during interfacial salting out chromatography on Sepharose 4B. The insert shows results of gel electrophoresis of Sephacryl S-200 Superfine peak (1); homogeneous L-leucine dehydrogenase (2); separated unknown proteins (3)

buffer, pH 7.5, containing 0.1% 2-mercaptoethanol. The enzyme was stored at 4° C or after addition of 50% glycerol at −20° C.

The purification of L-leucine dehydrogenase from 30 kg (wet weight) *Bacillus cereus* cells is summarized in Table 3.

Effect of pH on enzyme activity and stability

Optimal reaction rate for the oxidative deamination of L-leucine was found with glycine/NaCl/NaOH buffer at pH 11.5. The same result was obtained with L-valine and L-isoleucine as substrate. Below pH 10.5 the reaction rate decreased drastically in any case. The optimal pH for the reductive amination reaction depends, to some extent on the substrate employed. For 2-ketoisocaproate, 2-ketocaproate and 2-ketomethionine the maximal reactivity was found in the range of 9.0 to 9.5, whereas the optimal pH for 2-ketovalerate and 2-ketoisovalerate was found at pH 8.5 as shown in Fig. 5a and b. The pH-optima observed for both reactions are very similar to those of L-leucine dehydrogenase from *Bacillus sphaericus* (Ohshima et al. 1978).

The enzyme is stable in the pH range between 5.6 and 9.8 (Fig. 5c). At 25° C a maximal loss of enzymatic activity of 12% could be observed after 24 h storage. In the pH range of 6 to 7 there was no loss of activity when phosphate buffer was used. After 5 month we observed a loss of around 60% of the activity over the active pH-range tested. No loss of activity could be observed when glycerol was added to the enzyme solution to a final concentration of 50% after storage at −20° C for 1 year.

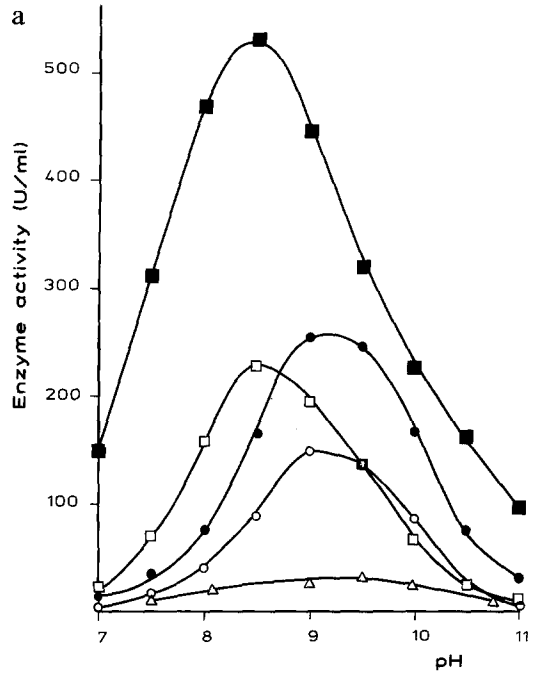
Effect of temperature on the enzymatic activity and stability

The influence of the assay temperature on the oxidative deamination reaction of L-leucine was

Table 3. Purification of L-leucine dehydrogenase

Step	Volume l	Protein g	Total activity U 10^{-3}	Specific activity U mg^{-1}	Yield %	Purification -fold
Crude extract	70	723	434	0.6	100	1
Heat treatment	70	145	421	2.9	97	4.8
Upper phase I	86	61	412	6.8	95	11.3
Lower phase II	99	40	373	9.3	86	15.5
Diafiltration	2.4	22	360	16.0	83	26.7
DEAE-Sephacell	4.7	13	308	24	71	40.0
Sephacryl S-200	0.48	6.3	260	41	60	68.3
Sepharose 4B	1.4	3.6	208	57	48	95.0

Oxidative deamination assay



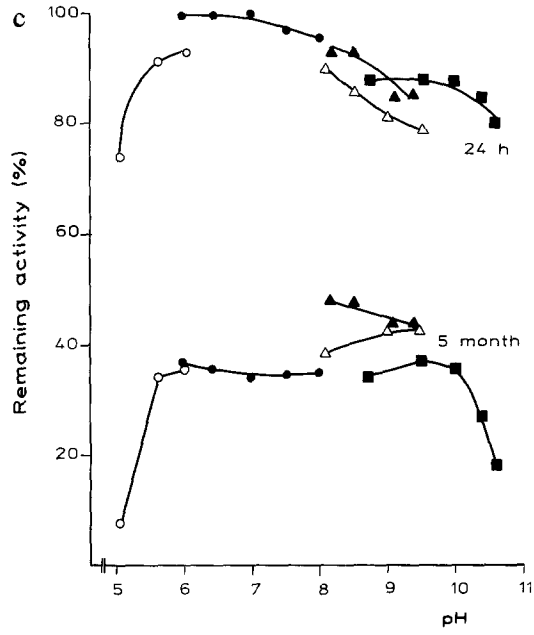
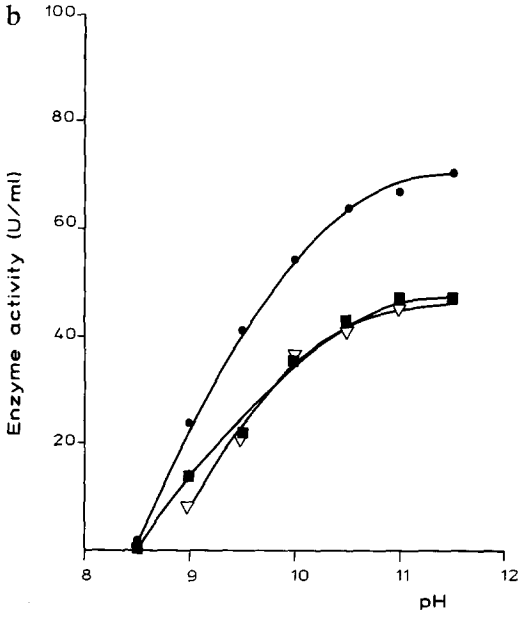


Fig. 5. Effect of pH on the activity of L-leucine dehydrogenase. **a** Reductive amination reaction; Substrates: 2-ketoisocaproate ●; 2-ketocaproate ○; 2-keto-4-mercaptobutyrate △; 2-ketoisovalerate ■; 2-ketovalerate □. **b** Oxidative deamination reaction; Substrates: L-leucine ●; L-valine ■; L-isoleucine ▽. The reaction rate for both reactions was measured as described in methods but with various substrates and at the pH-values indicated. The concentration of the substrates used gave optimal reaction rate under standard conditions. **c** The pH-stability of L-leucine dehydrogenase was tested by preincubation of the enzyme at 20° C for the time and the pH-values indicated. Buffers: sodium citrate ○; potassium phosphate ●; triethanolamine/NaOH △; Tris/HCl ▲; glycine/NaOH ■. The assay was carried out under standard conditions



measured at various temperatures from 25 to 73° C. The enzyme showed maximum activity at approximately 60° C as can be seen in Fig. 6. The heat stability of the enzyme was tested in crude extract and with purified enzyme. Both enzyme

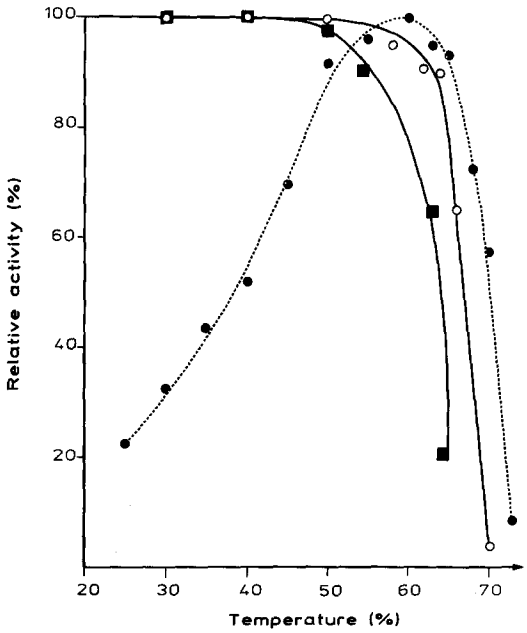


Fig. 6. Effect of temperature on the enzymatic activity and stability of L-leucine dehydrogenase. The oxidative deamination reaction of L-leucine was measured at various temperatures (●---●). The stability of the enzyme in crude extract (○—○) and as homogeneous protein (■—■) was tested by incubating in 100 mM phosphate buffer, pH 7.0, for 30 min at various temperatures. Then the enzyme solution was cooled and immediately assayed under standard conditions at 30° C

solutions were stable up to 50° C when heated 30 min in 50 mM potassium phosphate buffer (pH 7.8) containing 0.1% 2-mercaptoethanol. The stability of the purified enzyme decreased drastically above 55° C and above 64° C in crude extract as shown in Fig. 6. The difference observed may be a consequence of the protein concentration and/or the presence of substrates in the crude extract.

Coenzyme specificity

In the oxidative deamination reaction NAD^+ could not be replaced by $NADP^+$. Also NADH cannot be replaced by NADPH in the reverse reaction. The final concentration of $NADP^+$ and NADPH tested was 3.5 mM and 0.23 mM, respectively. The K_M-values for NAD^+ and NADH were estimated to be 0.34 mM and 0.034 mM, respectively.

Effect of metal ions and various compounds on the oxidative deamination of L-leucine

The effect of various compounds on leucine deamination is given in Table 4. The enzyme was incubated 10 min at 30° C with each compound (final concentration 1 mM) before starting the reaction with the coenzyme. Most of the compounds showed only weak effects on the enzymatic activity. On the other hand inhibition by $HgCl_2$, p-mercuribenzoate and KCN was observed. This inhibition suggests that L-leucine dehydrogenase contains a reactive sulfhydryl group(s).

Substrate specificity and kinetic constants

L-leucine dehydrogenase from *Bacillus cereus* catalyse the oxidative deamination of L-leucine, L-valine and L-isoleucine as well as straight-chain aliphatic amino acids as shown in Table 5. The reaction rate for the branched-chain amino acids was found much higher than with the straight-chain isomers. The K_M-values of the substrates tested are in the range of 1.0—2.9 mM, only with L-α-aminobutyrate and L-methionine the K_M-values increase by almost one order of magnitude. The substrate specificity of the enzyme for the reductive amination of various 2-ketoacids was also investigated and is summarized in Table 6. Branching at the C_3- or C_4-position results in an higher reaction rate than observed with straight-chain 2-ketoacids. Ammonia exhibited a rather high K_M-value about 0.22 M.

Table 4. Inhibition of L-leucine dehydrogenase

Compound [1 mM]	Relative activity [%]
None	100
$MgCl_2$	90
$MnCl_2$	94
$CaCl_2$	89
$CuSO_4$	95
$CoSO_4$	86
$ZnCl_2$	88
$HgCl_2$	8
EDTA	90
Citrate	91
1,10-Phenanthroline	81
2,2-Dipyridyl	91
Iodoacetamid	100
Iodoacetate	100
KCN	57
p-Mercuribenzoate	25
2-mercaptoethanol	85
Dithiothreitol	85
Reduced glutathion	92

Table 5. Substrate specificity of L-leucine dehydrogenase for the oxidative deamination

Substrate	V_{max} (Relative to L-leucine)	Optimal substrate concentration	K_M (M)
L-α-Aminobutyrate	24	$5 \times K_M$	2.2×10^{-2}
L-Norvaline	28	$7 \times K_M$	2.9×10^{-3}
L-Norleucine	6	$10 \times K_M$	1.5×10^{-3}
L-Valine	61	$3 \times K_M$	2.5×10^{-3}
L-Leucine	100	$6 \times K_M$	1.5×10^{-3}
L-Isoleucine	61	$6 \times K_M$	1.0×10^{-3}
L-Methionine	3	$6 \times K_M$	2.3×10^{-2}

Table 6. Substrate specificity of L-leucine dehydrogenase for the reductive amination

Substrate	V_{max} (relative to 2-keto-isocaproate)	Optimal substrate concentration	K_M (M)
2-Ketobutyrate	51	$10 \times K_M$	1.5×10^{-3}
2-Ketovalerate	51	$6 \times K_M$	4.0×10^{-4}
2-Ketocaproate	51	$6 \times K_M$	1.2×10^{-3}
2-Ketoisovalerate	154	$6 \times K_M$	2.1×10^{-3}
2-Ketoisocaproate	100	$10 \times K_M$	4.5×10^{-4}
2-Keto-3-methyl-valerate	107	$10 \times K_M$	9.0×10^{-4}
2-Keto-4-mercaptobutyrate	37	$5 \times K_M$	2.1×10^{-3}

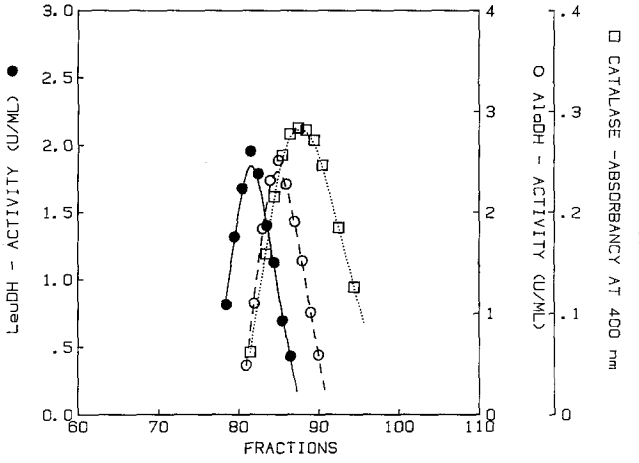


Fig. 7. Gel filtration on Sephacryl S-400 Superfine. Elution profile of L-leucine dehydrogenase ●—● and alanine dehydrogenase ○---○ from *B. cereus* and the marker protein catalase □ · · · □ (232000 MW)

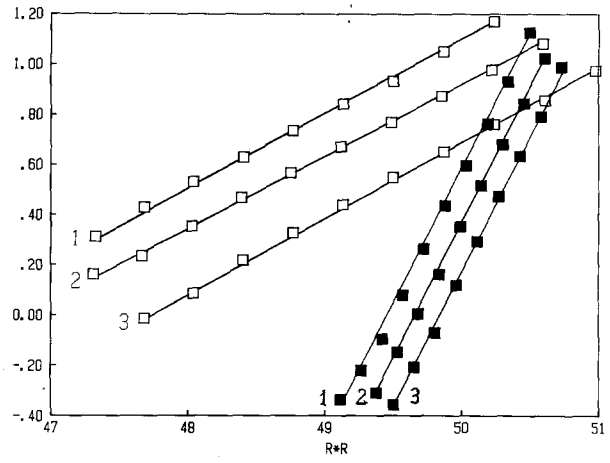


Fig. 8. Equilibrium sedimentation of L-leucine dehydrogenase at 6000 rpm (□—□) and 12000 rpm (■—■) at 8°C. Plot log optical density (o.D. 280 nm) versus r. The optical density of the different samples was: (1) 0.7; (2) 0.5 and (3) 0.3

Molecular weight and subunit structure

The molecular weight of the native enzyme was determined by gel filtration on Sephacryl S-400 Superfine and by high performance gel filtration on Superose 12. A linear calibration curve was obtained by plotting the log of molecular weight of the marker versus the elution volume. Using this curve a molecular weight of 310000 was estimated for the L-leucine dehydrogenase by both experiments. The good resolution of the Sephacryl S-400 superfine column in the molecular mass range 200—400 K dalton is demonstrated in Fig. 7. Gel electrophoresis of the enzyme in the presence of sodium dodecylsulfate showed only one band. The molecular weight was estimated as 39000 by comparison with a calibration curve obtained with standard proteins. Therefore it can be concluded the L-leucine dehydrogenase molecule is formed of 8 probably identical subunits.

Purified L-leucine dehydrogenase was sedimented to equilibrium at 6000 rpm and in another experiment at 12000 rpm. The temperature in both experiments was 8° C. The molecular weight was calculated from a plot on log absorption at 280 nm versus r^2 according to Svedberg and Pederson (1959). No dependence of the results could be detected in the range of 0.3 to 0.7 optical density as can be seen in Fig. 8. Assuming a partial specific volume of 0.732 ml/g which was calculated from the amino acid composition, we calculated a molecular weight of 310000 ± 10000.

The sedimentation coefficient was measured at 20° C and 60000 rpm from the same samples. The sedimentation coefficient appears to be independent of the enzyme concentration in this range. It was corrected for the viscosity and density of the solvent and calculated as $S_{w,20} = 11.65 \pm 0.1 \times 10^{-13}$ s (11.65 ± 0.1 S).

Crystallization of the enzyme

Purified leucine dehydrogenase can be crystallized readily from solution by addition of ammonium sulfate. The protein was dissolved (~10 mg/ml) in 0.01 M potassium phosphate buffer, pH 7.0, to which ammonium sulfate was added to 40% saturation. The turbid mixture was immedi-

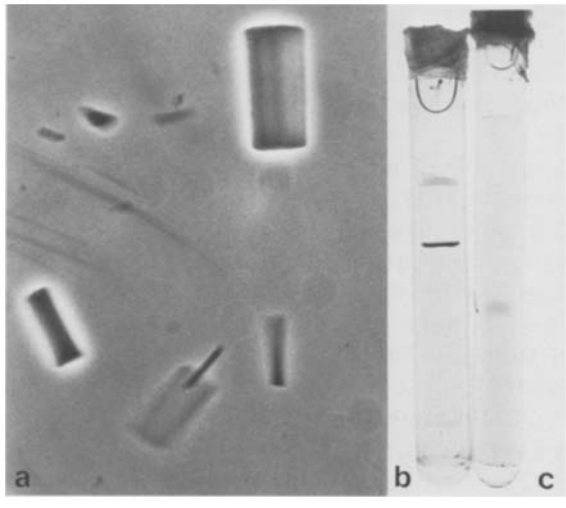


Fig. 9. Crystals of L-leucine dehydrogenase from *Bacillus cereus* (**a**) Electrophoresis of purified L-leucine dehydrogenase. Analytical acrylamide gel (**b**) and SDS-gel (**c**)

ately clarified by centrifugation. The enzyme crystals were obtained from the clean supernatant after being kept in cold room for a few days. Crystals and polyacrylamide gel electrophoresis of the purified enzyme are shown in Fig. 9.

Spectral properties

The purified enzyme when dissolved in 0.01 M potassium phosphate buffer, pH 7.0, gave an absorption maximum at 276 nm. No significant absorbance above 300 nm was observed. When the enzyme was dissolved in 6 M guanidinium hydrochloride at pH 12.5, the absorption maximum shifted to 295 nm due, mainly, to the ionization of the phenolic hydroxyl groups of tyrosine. Using the molar extinction coefficient of phenolate ion of tyrosine, $\Delta\varepsilon_{295\,nm}=2480$ (Edelhoch 1967), we calculated that there are about 17 tyrosine residues per subunit, which is in reasonable agreement with data obtained from amino acid analyses. Based on these results, we estimated that there is only 1 tryptophan per subunit.

Content of half-cystine

Spectrophotometric titration of sulfhydryl groups in the presence of 8 M urea indicated only 3 free SH-groups per subunit. Determination of cysteic acid using the performic acid oxidized enzyme revealed that there are about 5 cysteic acid residues in each subunit. Since the 310 K enzyme can be readely dissociated into 39 K subunits, we concluded that in each subunit there has to be one pair of half-cystine linked by a disulfide bond.

Amino acid composition

The complete amino acid composition of the enzyme is shown in Table 7. The data were calculated based on the assumption of 7 histidine residues in each subunit. It should be noted that the contents of glutamic acid and aspartic acid given are the sums of the free acid and the corresponding amides glutamine or asparagine. Furthermore, the data presented in Table 7 have not been corrected for loss, e.g. of serine by acid hydrolysis or incomplete release of valine and isoleucine, respectively. From the amino acid composition a molecular weight of 38000 was calculated for the subunit of leucine dehydrogenase in good agreement with data obtained by the other methods.

Number of tryptic peptides

As shown in Table 7, the enzyme contains about 40 arginine plus lysine residues per 38 K subunit. Hence, the 310 K native enzyme may contain totally 320 such residues. The fact that there were less than 30 tryptic peptides detectable by peptide mapping strongly indicate that the enzyme is composed of identical subunits. The number of tryptic peptides was actually lower than expected. This might be partly attributed to the presence of tandem sequences of lysine and/or arginine residues, and possibly also to the Lys-Pro and Arg-Pro sequences which are resistant to tryptic cleavage.

Table 7. Amino acid composition

Amino acid	Number of residues/38 K Subunit		
	24 h	48 h	72 h
Aspartic acid	41.5 (42)	38.8 (39)	38.2 (38)
Threonine	19.1 (19)	18.7 (19)	18.8 (19)
Serine	14.9 (15)	13.7 (14)	12.7 (13)
Glutamic acid	36.7 (37)	37.2 (37)	36.8 (37)
Proline	8.8 (9)	10.4 (10)	9.8 (10)
Glycine	34.0 (34)	32.6 (33)	33.1 (33)
Alanine	43.7 (44)	41.5 (42)	41.5 (42)
Half-cystine[a]	5.4 (5)	— (5)	— (5)
Valine	20.1 (20)	23.4 (23)	24.7 (25)
Methionine	5.1 (5)	4.7 (5)	4.7 (5)
Isoleucine	19.3 (19)	22.4 (22)	24.4 (24)
Leucine	23.5 (24)	24.0 (24)	24.3 (24)
Tyrosine	16.8 (17)	16.4 (16)	16.3 (16)
Phenylalanine	6.8 (7)	7.2 (7)	7.0 (7)
Histidine	7.0 (7)	7.0 (7)	7.0 (7)
Lysine	21.7 (22)	21.4 (21)	22.0 (22)
Arginine	19.8 (20)	18.6 (19)	17.9 (18)
Tryptophan[b]	(1)	(1)	(1)
Total	347	344	346

[a] Determined as cysteic acid
[b] Determined spectrophotometrically (Edelhoch 1967)

Discussion

Because of the high stereospecificity enzymes are useful for the synthesis of chiral compounds, such as L-amino acids. One of the enzymatic methods leading to L-amino acids is the reductive amination of corresponding 2-ketoacids with a specific NADH-dependent amino acid dehydrogenase such as alanine dehydrogenase (Wandrey et al.

1984b), phenylalanine dehydrogenase (Hummel et al. 1984) or leucine dehydrogenase (Wandrey et al. 1984c). By coupling formate dehydrogenase for regeneration of the coenzyme, the amination of 2-ketoacids can be conducted continuously in an enzyme-membrane reactor (Wandrey et al. 1984a). In this way L-leucine dehydrogenase has been employed for the synthesis of L-leucine as well as a variety of other straight- and branched-chain aliphatic amino acids. However, for industrial application of this process, the cost of the enzyme has to be minimized. This can be achieved, at least in part, by the improvement of the strain's productivity as well as by the development of an adequate process for enzyme recovery. *Bacillus sphaericus* and *Bacillus cereus* are known to be producers of L-leucine dehydrogenase. Our first goal was to improve the volumetric productivity for the enzyme. Especially a mutant constitutive for L-leucine dehydrogenase was sought for the development of a process to yield a high cell content per unit volume. Though we started our investigations of L-leucine dehydrogenase from *B. sphaericus* (Hummel et al. 1981) this strain proved to be less suitable for mutant selection. Among several isolates with high enzyme expression only one strain — *B. cereus* III/3 — showed continuous enzyme synthesis during the cultivation process. To gain high cell contents a fed-batch process was developed, using the pH as feeding parameter which proved to be simple and reliable for the enzyme production with *B. cereus*. Though application of this feeding strategy is so far not well documented in the literature (Yamane and Shimizu 1984). We consider this method to be suitable for technical application, in even larger scale than investigated here.

A large scale purification of L-leucine dehydrogenase was developed based on liquid-liquid extraction with aqueous two-phase systems (Kula et al. 1982). The enzyme was purified 16-fold with two partition steps. Compared with the conventional separation method the extraction procedure gives higher yield and higher specific activity of the enzyme, while the costs of the operation is considerably lower (Kroner et al. 1984; Schütte et al. 1983). We see other advantages of this technique in an easy scale-up and in the possibility of continuous operation (Hustedt et al. 1984). For final enzyme purification the interfacial salting-out chromatography on Sepharose-4B was choosen because of the good resolution and reproducibility as well as the high activity yield. Using this purification method the L-leucine dehydrogenase could be purified 95-fold with an overall yield of 48% and a specific activity of 57 U/mg (oxidative deamination). In *B. cereus* L-leucine dehydrogenase represents about 1% of the soluble protein content of the cells, it should be approximately 3% in the mutant *B. cereus* III/3. The final enzyme preparation could be crystallized and was homogeneous as judged by gel electrophoresis and ultracentrifugation studies. The molecular weight of L-leucine dehydrogenase from *B. sphaericus* was reported by Ohshima et al. (1977) to be about 245 000 consisting of six subunits with equal molecular weight of 41 000. From ultracentrifugation and gel filtration studies we found that the native enzyme from *B. cereus* has a molecular weight of 310 000 and is composed of 8 identical subunits with a mass of 39 000 each. On Sephacryl S-400 Superfine or Superose 12 the L-leucine dehydrogenase from *B. cereus* eluates well separated in front of the marker protein catalase (MW 232 000), whereas the L-leucine dehydrogenase from *B. sphaericus* elutes at the same position as catalase. The reason for this difference in quarternary structure of the L-leucine dehydrogenase from two *Bacillus* strains remains to be investigated. Enzyme from *B. cereus* also accepts molecular weight enlarged NADH (PEG 20 000-NADH), which is important for the application of the enzyme in an enzyme-membrane-reactor. L-leucine dehydrogenase from *B. cereus* has been repeatedly and successfully applied in enzyme-membrane reactors for periods of 3 month with a consumption of less than 100 U per kg L-leucine produced, which means that the enzyme purified here is sufficient to produce at least 2 tons of the L-amino acid (C. Wandrey, W. Leuchtenberger, private communication).

Acknowledgements. The authors are grateful to Mrs. R. Getzlaff, Miss S. Löslein and Mrs. A. Ziehr and Mr. R. Kraume-Flügel for their expert technical assistance during various stages of the work.

We thank also Dr. J. Flossdorf and Mr. H. Schilling for measuring ultracentrifugal data.

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Received April 25, 1985