Screening for Amidases: Isolation and Characterization of a Novel D-Amidase from *Variovorax paradoxus*

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Dedicated to Professor Dr. Roger A. Sheldon on the occasion of his 60th birthday.

Abstract: Using racemic *tert*-leucine amide as sole nitrogen source in minimal medium, 162 strains were isolated by enrichment techniques and shown to contain amidase activity. Among these isolates three D-amidase producers were found and identified as *Variovorax paradoxus* (two strains) and *Klebsiella* spec. The D-amidase from *Variovorax paradoxus* was purified to homogeneity by three chromatographic steps. With DL-Tle-amide as substrate Michaelis–Menten kinetics were observed with a K_M of 0.74 mM, a K_I of 640 mM and a V_{max} of 1.4 U/mg. The amidase has a broad pH-optimum between 7 and 9.5 and a temperature optimum at 47–49 °C. The amidase hydrolyzed amino acid amides as well as carboxamides and 2-hydroxy acid amides. The stereoselectivity of the reaction was variable, however. Hydrolyzing DL-Tle-amide the enantiomeric ratio E was >200 resulting in D-Tle with an ee of >99% and up to 47% conversion. Similar results were obtained with DL-Leu-amide and DL-Val-amide while DL-Phe-amide was hydrolyzed with an enantiomeric ratio E of only 5.

Keywords: D-amidase; D-amino acids; enzyme screening; *tert*-leucine; *Variovorax paradoxus*

Introduction

The production of chiral 2-amino acids was an important driving force in the development of enzyme-catalyzed processes. L-2-Amino acids are the building blocks of all proteins and peptides and therefore an essential component of human and animal nutrition. Special amino acids – often with sterically demanding side chains – are important intermediates in the synthesis of enzyme inhibitors or receptor blockers.[1] D-Amino acids are widely used to synthesize derivatives of penicillin or cephalosporin with improved pharmaceutical properties.[2] Furthermore, chiral amino acids or their derivatives are employed as catalysts[3] and chiral auxiliaries in chemical asymmetric synthesis[4] or as ligands in the development of new organometallic catalysts.[5] Chiral 2-amino acids are produced by exploiting different enzymes and precursors, e.g.:

- enantioselective hydrolysis of racemates starting with *N*-acetylamino acids, amino acid amides or hydantoins,
- stereoselective addition of ammonia to double bonds,
- modification of chiral precursors,
- transamination or reductive amination of 2-oxo acids.[6]

The latter reaction is used for the industrial production of L-*tert*-leucine [L-Tle, (*S*)-2-amino-3,3-dimethylbutanoic acid],[7] utilizing leucine dehydrogenase (EC 1.4.1.9) from *Bacillus cereus* as catalyst and regenerating NADH *in-situ* by formate dehydrogenase (EC 1.2.1.2), see Figure 1.[8] Several other L-amino acids with sterically demanding side chains can be produced by the same route.[9]

Only L-enantiomers are accessible by reductive amination since D-selective amino acid dehydrogenases have not been described until now. There is a high interest to make also D-Tle and related D-amino acids available by a feasible approach.

Recently, an asymmetric catalytic variant of the Strecker reaction has been described yielding D-Tle in high enantiomeric purity (ee >99%).[10] Whether larger

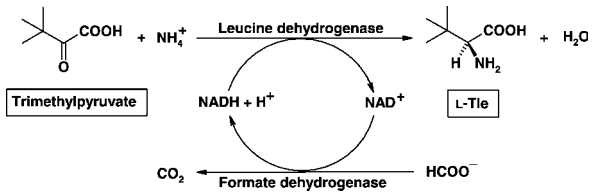

Figure 1. Synthesis of L-*tert*-leucine via reductive amination.

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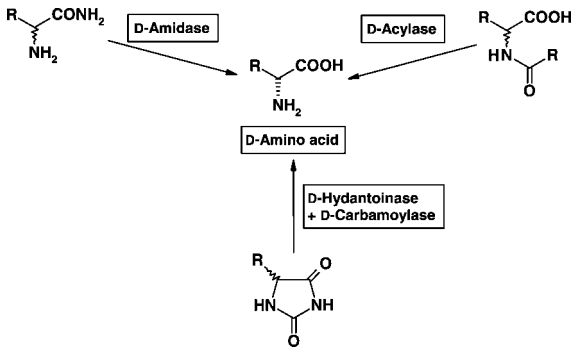

Figure 2. Possible enzymatic routes for kinetic resolutions of amino acid precursors with D-specific hydrolases.

amounts can be produced this way remains to be established.[11]

Enzymatic approaches for the production of D-Tle have also been investigated. The commercially available acylases and amidases do not accept the corresponding Tle derivatives as substrates, however. Using whole cell biocatalysts activity towards L-Tle-NH_2 was observed in some cases.[12,13] With a D-hydantoinase (EC 3.5.2) it was possible to stereoselectively open the ring of racemic 5-*tert*-butylhydantoin. The resulting *N*-carbamoyl-D-Tle can be converted to D-Tle using a D-carbamoylase (EC 3.5.1.77) as a second enzyme or by a chemical reaction with nitrite.[7] 5-*tert*-Butylhydantoin is a very poor substrate of the available hydantoinase, therefore, long reaction times are required, which make this route less attractive for a larger scale. To provide a better alternative, we decided to screen for amidases (EC 3.5.1.4) or acylases (EC 3.5.1.14) able to accommodate structures in their reactive sites, which are highly branched in the C-3 position, like Tle (Figure 2).[14]

Since such enzymes appear quite rare we employed an enrichment technique to find enzyme producers able to utilize racemic precursors of Tle as carbon and/or nitrogen source. A similar approach had proven successful before, e.g., in the isolation of carnitine amidase producers[15] or D-amidase producers.[16–18] In this paper we describe the screening for Tle amidase producers, as well as the isolation and characterization of a new D-amidase.

Results and Discussion

Screening

We decided to use DL-*N*-acetyl-Tle-amide as screening substrate first to avoid nitrogen mobilization by transamination and thus the generation of false positive colonies. A microbial peptide amidase[19] has been described hydrolyzing *N*-acetylamino acid amides[20] stereoselectively, though most known amino acid amidases require a free amino function for substrate recognition.[21] When DL-*N*-acetyl-Tle-amide was added to medium (M1) as nitrogen source, a few strains were obtained after the enrichment procedure growing poorly. However, none of the isolated strains exhibited amidase activity towards DL-*N*-acetyl-Tle-amide. The negative result could be a consequence of the repressor activity due to the glucose employed as the carbon source. Attempts to employ DL-*N*-acetyl-Tle-amide as single carbon and nitrogen source (medium M2) were hampered by the unexpectedly low solubility of the screening substrate ($<0.5\%$) and no amidase producer was obtained from enrichment cultures.

In the next enrichment procedure, we used DL-Tle-amide as nitrogen source (medium M3), which proved much more productive. Out of 81 samples collected from soil/water/natural habitats, 224 single colonies were isolated and tested for amidase activity. Among those, 62 strains were discarded, because of their poor growth in liquid media. The remaining 162 strains (72%) revealed amidase activity when tested for ammonia liberation from DL-Tle-amide. Using a chiral thin-layer chromatography for amino acids the enantioselectivity was determined as high D- or L-selective (ee $>80\%$) or non-selective amidase activity. Analysis of the positive strains revealed 3 strains that produced the desired D-amidase, while all others produced predominantly an L-amidase. The screening results are summarized in Table 1. Apparently, transaminases do not tolerate substrates highly branched at C-3 as all isolated strains, which grew sufficiently in liquid minimal medium, exhibited amidase activity. Since L-amino acids are predominantly found in nature, it is not a surprising result that the majority of the isolates had an L-amidase. Among the previously described amidases only a few are D-selective enzymes and the majority of these were obtained by screening on the corresponding D-enantiomer.[16–18]

The three isolated D-amidase producers were identified by the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). The results as well as some activity data are shown in Table 2. The two *Variovorax* strains originated from different environmental samples. Since isolate 19-3 had a significantly higher specific activity in the crude extract, it was selected for further studies.

Table 1. Results of screening 224 strains isolated from enrichment cultures of 81 samples from soil/water using DL-Tle-amide as nitrogen source.

Strains	Number	[%]
Isolated	224	100
Amidase activity	162	72
L-amidase	159	71
D-amidase	3	1.3

Table 2. Identity of the isolated D-amidase producers. Activity was determined in the crude extract after growth of the strains in medium M3.

Strain No.	Identity	Activity [mU/mL]	Spec. activity [mU/mg]
19-3	*Variovorax paradoxus*	64	33
34-4	*Variovorax paradoxus*	38	19
47-4	*Klebsiella* sp.	<5	–

Cultivation of *Variovorax paradoxus*

Growth of the *Variovorax* strain on medium M3 was rather slow, after about 90 h the stationary phase was reached at an optical density at 660 nm of about 2. The specific activity of the D-amidase was almost constant throughout the growth period. Furthermore, in this minimal medium the strain produced much slime, presumably because of the unfavorable C/N ratio.[22] Growth on media recommended by the DSMZ catalogue (medium 1 and 81) for *Variovorax* strains was faster but resulted in about the same optical density (2.5) and no D-amidase could be detected in the absence of DL-Tle-amide as inducer. Addition of yeast extract in the minimal medium M3 accelerated the growth rate but gave less D-amidase activity. Attempts to induce the amidase production with other commercially available amides, such as isobutyramide, instead of DL-Tle-amide also failed, as the cell yield increased about 100% but the specific activity of the crude extract dropped to <3 mU/mg. Therefore, 30 L minimal medium M3 was employed in a bioreactor to produce enough biomass for the purification and characterization of the D-amidase. On this scale an average cell yield of approximately 5 g (wet weight)/L culture volume and a specific activity of 20 mU/mg was reached. The activity yield was about 1 U/L culture volume.

Purification of the D-Amidase from *Variovorax paradoxus*

After some initial optimization, the D-amidase was purified by three chromatographic steps as summarized in Table 3. The low yield after the last step resulted from losses during ultrafiltration using YM 10 membranes to concentrate the pool. Later, it was found that losses could be minimized by using Omega membranes instead.

After the HIC-step on butyl-Sepharose, the D-amidase was about 50% pure and contaminated by only one other protein as shown in Figure 3. The contaminating protein could be completely removed by gel filtration yielding the homogeneous enzyme. From the gel filtration, the native molecular weight of the D-amidase was determined as 171 kDa, while the molecular weight of the denatured protein chain was determined as 50 kDa by SDS-PAGE (Figure 3). However, additional information is required to establish the quaternary structure of the D-amidase.

Characterization of the D-Amidase from *Variovorax paradoxus*

Influence of Metals and Inhibitors on the Amidase Activity

Mg^{2+}, Ca^{2+}, Zn^{2+}, Mn^{2+}, Co^{2+} and Ni^{2+} were tested for possible enzyme activation but showed no effects. Also,

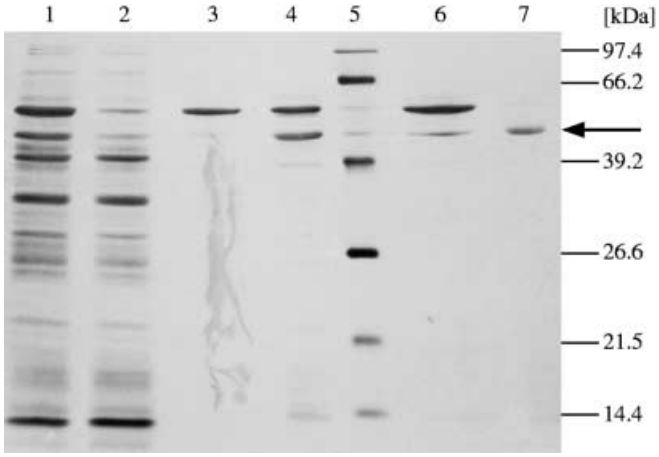

Figure 3. Purification of D-amidase from *Variovorax paradoxus* after HIC, analyzed by SDS-PAGE: IEC-pool (lane 1; 5 μg protein), flow through HIC (lane 2; 5 μg protein), fraction No. 6: 0.07 U/mg (lane 3, 2.5 μg protein), fraction No. 9: 0.2 U/mg (lane 4, 2.5 μg protein), marker proteins (lane 5), fraction No. 13: 0.8 U/mg (lane 6, 2.5 μg protein), fraction No. 17: 1.4 U/mg (lane 7, 2.5 μg protein). D-Amidase is indicated with an arrow. The gel was silver stained.

Table 3. Purification of D-amidase from 80 g wet cells from *Variovorax paradoxus*.

Purification step	Spec. activity [U/mg]	Protein [mg]	Total activity [U]	Yield [%]	Purification factor
Crude extract	0.02	800	16	100	–
Q-Sepharose FF	0.16	94	15	94	8
Butyl-Sepharose FF	0.68	20	14	87	7
Superdex 200 PG	1.4	2.9	4	25	2

Table 4. Effects of various compounds on the D-amidase from *Variovorax paradoxus*. Enzyme preparations after two chromatographic steps were used with a specific activity of 0.56 U/mg.

Additive	Relative activity [%][a]	
	Additive (1 mM)	Additive (0.1 mM)
none (control)	100	100
Hg^{2+}, Ag^{+}	0	0
Cu^{2+}	1.3	88
Fe^{2+}	69	92
Fe^{3+}	100	97
Pb^{2+}	104	100
phenylmethanesulfonyl fluoride	0	6.9
p-chloromercuribenzoate	0.7	38
DTNB[b]	79	90
iodoacetamide	93	101
hydroxylamine	100	100
EDTA	104	n. d.
o-phenanthroline	99	n. d.

[a] After preincubation of the enzyme for 30 min with the compound to be tested, enzyme activity was measured by HPLC under the standard conditions described in the experimental section.
[b] 5,5′-Dithiobis-(2-nitrobenzoic acid), DTNB.

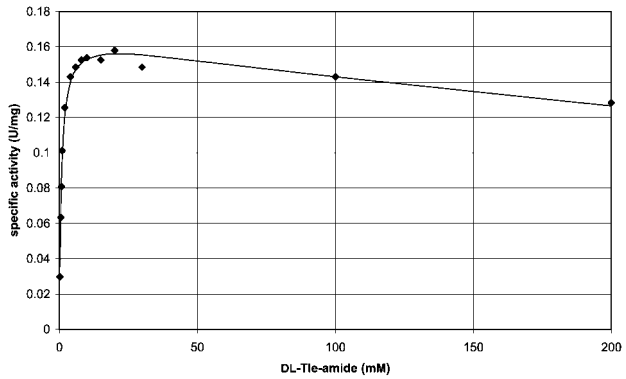

Figure 4. Michaelis–Menten plot for the conversion of DL-Tle-amide by the D-amidase from *Variovorax paradoxus*. Enzyme preparations after one chromatographic step were used with a specific activity of 0.16 U/mg.

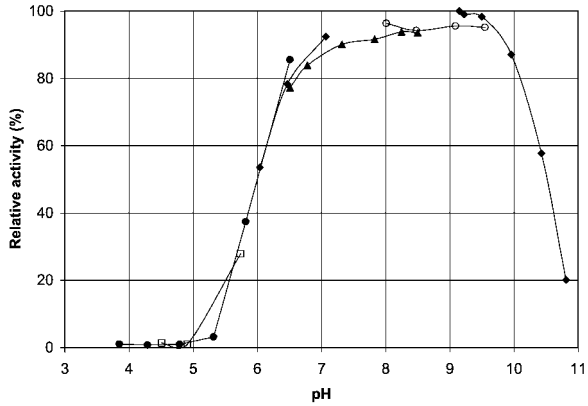

Figure 5. Optimum pH for the D-amidase activity. Levels of activity were determined by using the standard assay and the following buffers (each at a final concentration of 0.1 M): sodium citrate (●), sodium acetate (□), 2-(*N*-morpholino)ethanesulfonic acid (MES) (◆), potassium phosphate (▲), Tris-(hydroxymethyl)aminomethane (○), and sodium carbonate (◆). The activity for sodium carbonate buffer, pH 9.2, with 0.61 U/mg was taken as 100%.

the D-amidase was not inactivated by chelators such as EDTA and *o*-phenanthroline (Table 4). Both results demonstrate that the amidase does not require metal ions as a cofactor. The other results summarized in Table 4, indicate that the D-amidase may have a serine in the catalytic center as indicated by the inhibition with phenylmethanesulfonyl fluoride known to interact with serine in the active center of proteases.[23] Cysteine(s) appears to play an important role also for activity and/or structural integrity of the enzyme. The latter is referred from the strong inhibition by the sulfhydryl reagent *p*-chloromercuribenzoate and the metal ions Hg^{2+}, Ag^{+} and Cu^{2+} at 1 mM concentration.

Kinetics

With partially purified D-amidase (after ion exchange chromatography) the initial rate of hydrolysis was determined. The results are shown in Figure 4. The kinetics can be described well by the Michaelis–Menten equation taking into account a slight substrate inhibition. Thus for DL-Tle-amide a K_M of 0.74 mM and a K_I of 640 mM were calculated. Pure amidase reached a V_{max} of 1.4 U/mg.

The pH profile of the D-amidase is presented in Figure 5. It shows a broad optimum from pH 7 to 9.5.

Table 5. Substrate specificity of partially purified D-amidase for amino acid amides and 2-hydroxy acid amides. Enzyme preparations after two chromatographic steps were used with a specific activity of 0.56 U/mg.

Substrate	Relative activity[a, b]
DL-Tle-amide	1
D-Val-amide	13
L-Val-amide	1.2
D-Leu-amide	400
L-Leu-amide	26
L-Ile-amide	0.35
D-Ala-amide	44
L-Ala-amide	15
D-Phe-amide	890
L-Phe-amide	190
L-Tyr-amide	100
DL-Trp-amide	160
L-Trp-amide	62
DL-Met-amide	230
L-Met-amide	86
D-Pro-amide	24
L-Pro-amide	56
D-Lactic acid amide	59
L-Lactic acid amide	66
DL-HMB-amide [c]	150

[a] The activity was determined by HPLC at a substrate concentration of 20 mM. The value for DL-Tle-amide (0.56 U/mg) was taken as 1.
[b] The activity for proline amides and the hydroxy acid amides were determined from the amount of liberated ammonia.
[c] DL-HMB-amide = DL-2-hydroxy-4-methylthiobutanoic acid amide.

Table 6. Substrate specificity of partially purified D-amidase for carboxamides. Enzyme preparations after two chromatographic steps were used with a specific activity of 0.56 U/mg.

Substrate	Relative activity[a]
DL-Tle-amide	1
Formamide	0.13
Acetamide	4.0
Propionamide	26
n-Butyramide	37
Isobutyramide	33
Valeric acid amide	36
Acetoacetic acid amide	130
Malonic acid diamide	2.9
Succinic acid diamide	2.1
Adipic acid diamide	14
Acrylamide	7.7
Benzamide	5.2
Nicotinamide	19
L-Asparagine	0.15
D-Asparagine	<0.05

[a] The activity was determined from the amount of liberated ammonia, and the value for DL-Tle-amide (0.56 U/mg) was taken as 1.

The temperature optimum for incubation times of 15 min was found at 47 – 49 °C (data not shown).

Substrate Range

Besides various amino acid amides, 2-hydroxy acid amides, carboxamides and *N*-acylated amino acid amides were investigated as substrates for the D-amidase using a partially purified enzyme (HIC pool). The results are summarized in Table 5 and 6.

If available, both enantiomers of the amino acid amides were used in the study. With the exception of proline, HPLC was used to quantify the amino acids. The hydrolysis of proline amides and the hydroxy acid amides was determined from the liberated ammonia. Not unexpected was the observation that the activity of the D-amidase was higher towards other substrates than DL-Tle-amide. With 500 U/mg the highest activity was found towards D-Phe-amide, which is a 890-fold increase compared to DL-Tle-amide. From the series Leu > Val > Tle it can be seen that branching at C-3 of amino acid amide substrates has a strong influence on the activity. In general, the D-enantiomers are the preferred substrates. With proline the L-enantiomer is hydrolyzed faster, however, and the L- and D-enantiomers of lactic acid amide are hydrolyzed with nearly equal rates.

The D-amidase from *Variovorax paradoxus* also readily hydrolyzes carboxamides. The highest activity was observed with acetoacetic acid amide. Whether the amides of the dicarboxylic acids were hydrolyzed stepwise cannot be decided with the assay used. The corresponding amino acid, asparagine, containing an amide function in the γ-carboxyl group, was a very poor substrate; compared to DL-Tle-amide it was converted with 15% activity only.

As expected, *N*-acetylated amino acid amides were very poor substrates, if hydrolyzed at all (data not shown).

Enantioselectivity

The optical purity of D-Tle obtained during hydrolysis of the racemic substrate by the D-amidase was measured as a function of conversion. The results are summarized in Table 7. It can be seen that up to 47% conversion the ee value of D-Tle is > 99. At the theoretical maximum yield of 50% the ee value of the product was still 97.1% and dropped further upon prolonged incubation. The enantiomeric ratio E[24] well above 200 demonstrated the high preference of the D-amidase from *Variovorax* for D-Tle-amide as substrate. With the enzyme pool

Table 7. Enantioselectivity of the D-amidase as a function of conversion c of DL-Tle-amide. Enzyme preparations after two chromatographic steps were used with a specific activity of 0.56 U/mg.

Conversion c[a] [%]	$ee_{D\text{-}Tle}$[b] [%]
18	98.8
29	99.0
40	99.0
43	99.2
45	99.4
47	99.1
48	98.6
50	97.1

[a] Conversion c = (D-Tle + L-Tle)/(DL-Tle-amide$_{t=0}$) × 100.
[b] $ee_{D\text{-}Tle}$ = (D-Tle − L-Tle)/(D-Tle + L-Tle) × 100. ee, enantiomeric excess.

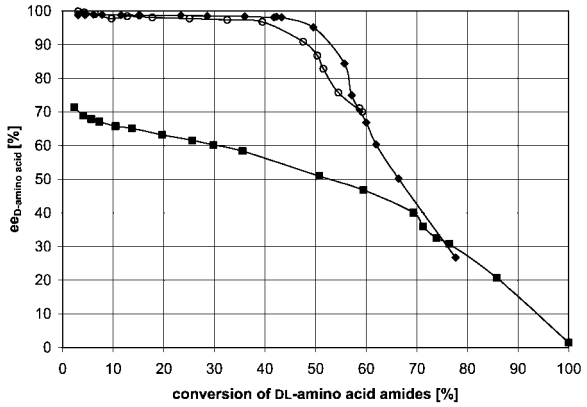

Figure 6. Enantioselectivity of the D-amidase as a function of conversion c of DL-Val-amide (◆), DL-Leu-amide (○) and DL-Phe-amide (■).

obtained after the ion exchange chromatography the enantiomeric ratio E was slightly lower, at 120–180 yielding product at 50% conversion with 96.5% ee (data not shown). The results suggest that partially purified enzyme preparations can be used successfully for the resolution of the racemate.

The enantioselectivity of the amidase is also high for Leu and Val, which was demonstrated by measuring the ee values as a function of conversion (Figure 6). D-Phe-amide, the substrate with the highest hydrolysis rate (500 U/mg) had an E value of only 5, resulting in an ee of 50.9 at 51% conversion.

Regarding the enantioselectivity for D-amino acid amides and the described wide substrate range, the D-amidase from *Variovorax paradoxus* appears to be different from other well-known amidases.[14] Similar to the D-amino acid amidase from *Ochrobactrum anthropi* SV3[16] and the (*R*)-ketoprofen-amidase from *Comamonas acidovorans* KPO-2771-4,[25] the new enzyme has high activity for D-Phe-amide. The D-amino acid amidase from *Ochrobactrum* did not hydrolyze aliphatic carboxamides, like acetamide and propionamide. Whether these results described here for the D-amidase from *Variovorax paradoxus* are due to the second protein in the enzyme preparation remains to be confirmed. The (*R*)-ketoprofen-amidase hydrolyzed Phe-amide, Leu-amide and Gly-amide among 5 amino acid amides tested. The D-alanine acid amidase from *Arthrobacter sp.* NJ-26[26] and the D-aminopeptidase from *Ochrobactrum anthropi* C1-38[17] are enzymes specific for D-Ala-amide and D-Ala-peptides and amino acid amides with larger side chains were not hydrolyzed. The physiological substrate for the enzyme from *Variovorax paradoxus* remains to be elucidated.

Conclusion

In a screening with DL-Tle-amide as sole nitrogen source a strain of *Variovorax paradoxus* was isolated producing an inducible D-amidase, which is a promising enzyme for the production of D-Tle by stereoselective resolution of the racemate with an E value of >200. The enzyme had the highest stereoselectivity towards aliphatic amino acid amides with a chain length of about 5 carbon atoms. At present only small amounts of the D-amidase can be produced with the wild type organism *Variovorax paradoxus*. Work is in progress, however, to clone and express the gene in a recombinant host. The D-amidase should be available soon in sufficient quantities for further process development.

Experimental Section

Materials and General Methods

DL-Tle-amide, L-Tle-amide, *N*-acetylamino acid amides, *N*-acetylamino acids, L-Tle, DL-2-hydroxy-4-methylthiobutanoic acid amide (DL-HMB-amide) were kindly supplied by Degussa AG (Hanau-Wolfgang, Germany). Other amino acid derivatives were purchased from Novabiochem (Bad Soden, Germany) or Bachem Biochemica (Heidelberg, Germany). NADH, 2-oxoglutarate and adenosine diphosphate were products from Sigma-Aldrich (Deisenhofen, Germany), and the glutamate dehydrogenase was obtained from Fluka (Deisenhofen, Germany). All other chemicals unless noted otherwise were commercial products of the highest purity available. Media components were obtained from Merck (Darmstadt, Germany) or Difco (Becton Dickinson, Sparks, USA)

Culture Media

Media sterilization was carried out at 121 °C at 1.2 bar for 20 min. Glucose, $MgSO_4 \cdot 7\,H_2O$, $CaCl_2 \cdot 2\,H_2O$, Tle-derivatives, vitamin solution and trace element solution were added separately after cooling of the medium by sterile filtration

using 0.2 μm membranes Minisart NML (Sartorius, Göttingen, Germany). For solid media 1.5% agar (w/v) was added. The minimal medium M1 comprised 1.95 g KH_2PO_4, 2.5 g K_2HPO_4, 1.0 g NaCl, 0.3 g $MgSO_4 \cdot 7\,H_2O$, 0.05 g $CaCl \cdot 2\,H_2O$, 2.0 g glucose, 2.0 g DL-*N*-acetyl-Tle-amide, 0.8 mL trace element solution and 2.5 mL vitamin solution in 1000 mL. In minimal medium M2 glucose was omitted, for the rest it had the same composition as given for M1. Minimal medium M3 contained 3.3 g KH_2PO_4, 0.8 g K_2HPO_4, 1.0 g NaCl, 0.3 g $MgSO_4 \cdot 7\,H_2O$, 0.05 g $CaCl_2 \cdot 2\,H_2O$, 4.5 g glucose, 3.3 g DL-Tle-amide, 0.8 mL trace element solution and 2.5 ml vitamin solution in 1000 mL. The trace element solution was prepared as described by Joeres and Kula,[15] the composition of the vitamin solution was taken from Schlegel.[27]

Screening Procedure

30 mL of a minimal medium containing either DL-*N*-acetyl-Tle-amide as nitrogen source (M1) or as carbon and nitrogen source (M2) or DL-Tle-amide as nitrogen source (M3) was inoculated with a sample from natural water sources or a suspension of soil from various habitats in sterile saline. The shake flasks with the cultures were placed on a rotary shaker operated at 150 rpm and 30 °C. An aliquot was taken after 3 – 7 days depending on the developing turbidity indicating microbial growth, and transferred into 30 mL of fresh medium. After 4 transfers the cultures were diluted and plated on agar plates prepared with the same medium and incubated at 30 °C. The development of colonies was observed by visual inspection over several days. Up to four single colonies from each plate were picked and purified by further plating until pure cultures were obtained. Pure cultures were maintained on agar plates with minimal medium at 4 °C and grown in 30 or 100 mL liquid media in shake flasks for further evaluation.

Cultivation of Variovovax paradoxus

To obtain sufficient biomass for enzyme isolation the D-amidase producer was cultivated in 30 L medium M3 in a bioreactor (Techfors, 40 L reactor volume, Infors, Bottmingen, Switzerland) for 92 h. Cells were harvested using a chamber centrifuge KA 02 (Westfalia Separator, Oelde, Germany) operated at 9600 rpm. Cells were frozen and stored at −20 °C until further use.

Cell Disintegration

Wet cells were suspended in 0.1 M potassium phosphate (KPi buffer) pH 7.5 to 20% (w/v) and disintegrated by wet milling using 0.3 mm glass beads and a mixer mill (Retsch, Haan, Germany) for small volumes[28] or the disintegrator S (IMA, Frankfurt, Germany) operated at 3500 rpm, for 20 min. Glass beads and cell suspension were mixed in a ratio 2:1. Thereafter, cell debris and glass beads were sedimented by centrifugation and the supernatant was used as the enzyme source.

Analytical Methods

Amidase Assay

For the amidase assay, incubation was carried out at 30 °C in a total volume of 500 μL containing 400 μL 0.1 M KPi, pH 7.5, 50 μL substrate (0.2 M DL-Tle-amide) and 50 μL crude extract. After 10 – 60 min incubation the reaction was terminated by heating the mixture for 3 min at 95 °C. Precipitated protein was centrifuged off at 13000 rpm for 5 min. Two control reactions were included, in which the hydrolysis reaction was performed in the absence of substrate or enzyme extract, respectively. Aliquots of the supernatant were either analyzed for ammonia generated by the amidase activity or for Tle by an HPLC method. One unit amidase activity hydrolyzed 1 μmole Tle-amide per minute under the specified conditions.

Ammonia was determined by the glutamate dehydrogenase assay as described by Bergmeyer and Beutler[29]. The method is fast but does not provide information on the stereoselectivity.

HPLC for the Determination of D- *and* L-*Amino Acids*

To separate amino acid enantiomers by RP-HPLC and enable their detection by fluorescence, they were converted into diastereomers using *N*-isobutyryl-L-cysteine (IBLC) or *N*-isobutyryl-D-cysteine (IBDC) as chiral reagents together with *o*-phthaldialdehyde (OPA) according to Brückner et al.[30] The derivatization reagent was prepared in 100 mM sodium borate buffer pH 10.4 containing 170 mM OPA and 260 mM IBLC or IBDC. 20 μL reagent were mixed with 180 μL sample diluted in 100 mM sodium borate buffer pH 10.4. An aliquot of 20 μL was used for analysis. As stationary phase, a Kromasil column (250 × 4 mm, 5 μm, 100 Å) was employed in HPLC equipment (Dionex, Germering, Germany). For separation, a two component buffer was applied consisting of A: 23 mM sodium acetate, titrated with acetic acid to pH 6.0 and B: acetonitrile mixed with water 10:1.5 (v/v).[31] The Tle, Leu, Ile, Val, Phe, Tyr, Trp und Met enantiomers were separated using a flow rate of 1 mL/min and the following gradient: time t = 0 min 22% buffer B, 25 min 29% B, 27 min 100% B, 30 min 100% B, 32 min 0% B, 42 min 0% B. For Gln, His and Ala the gradient program had to be changed starting with 12% buffer B (Gln and His) and 15% B (Ala) for sufficient separation of the enantiomers. The corresponding amides were also modified and eluted well separated from the free amino acid. A fluorescence detector was used with the excitation wavelength set at 340 nm and emission at 440 nm. The peak area was calibrated using the pure enantiomers whenever available. Optical purity was expressed as: enantiomeric excess (% ee) = (major enantiomer – minor enantiomer)/(sum of enantiomers) × 100. The enantiomeric ratio E was calculated as: $E = \ln[1 - c(1 + ee_{D\text{-Tle}})]/\ln[1 - c(1 - ee_{D\text{-Tle}})]$.[21]

Chiral Thin-Layer Chromatography (TLC)

For qualitative evaluation of enzyme selectivity during screening chiral thin-layer chromatography (CHIRAL-PLATE silica gel RP modification coated with Cu^{2+} and chiral reagent; Macherey and Nagel, Düren, Germany) was employed and methanol/water/acetonitrile 1:1:4 (v/v/v) was used as the mobile phase. Plates were activated for 15 min at 100 °C prior

to use. After development plates were dried and spots visualized by reaction with ninhydrin (0.3% (w/v) ninhydrin in 2-methylpropanol).

Protein Determination

Total protein was determined according to Bradford.[32] Bovine serum albumin (BSA) was used for calibration.

Purification of D-Amidase from *Variovorax paradoxus*

Step 1. Ion Exchange Chromatography (IEC): Q-Sepharose FF (Amersham Biosciences, Freiburg, Germany) was employed for the first step during purification. The column (5 × 21 cm, 410 mL volume) was equilibrated with 20 mM KPi buffer, pH 6.5 (buffer A), and operated with a flow rate of 10 mL/min. A sample of 185 mL crude extract was applied to the column, and after washing elution was carried out in a linear gradient changing to buffer B (20 mM KPi buffer, pH 6.5, 150 mM sodium sulfate) within 4 column volumes. Fractions of 12 mL were collected and analyzed for activity. The appropriate fractions were pooled and adjusted to a sodium sulfate concentration of 150 mM measuring the conductivity (25 mS/cm).

Step 2. Hydrophobic Interaction Chromatography (HIC): A column with 6.5 mL Butyl-Sepharose FF (Amersham Biosciences, Freiburg, Germany) was equilibrated with 20 mM KPi buffer, pH 6.5, 150 mM sodium sulfate before the pool of the anion exchange column was applied at a flow rate of 1 mL/min. Elution was performed in a linear gradient decreasing the sodium sulfate concentration to zero within 6 column volumes. Fractions of 4 mL were collected. Fractions containing D-amidase activity were pooled and concentrated by ultrafiltration.

Step 3. Gel Filtration (GPC): Superdex 200 PG (Amersham Biosciences, Freiburg, Germany) was packed in a column (1.6 × 62 cm, 124 mL volume) in 20 mM KPi buffer, 180 mM NaCl. The column was operated with a flow rate of 1 mL/min in isocratic mode. The effluent was analyzed at 280 nm and 1 mL fractions collected. Aliquots (1.0 mL) of the pool from the HIC column were applied to achieve the final purification of the D-amidase. The column was calibrated using the proteins from the low and high molecular weight Kit (Amersham Biosciences, Freiburg, Germany).

Ultrafiltration: Diluted enzyme fractions were concentrated by ultrafiltration using YM 10 membranes (Millipore, Eschborn, Germany) or Omega membranes (Pall Gelman Laboratory, Dreieich, Germany), both membranes have a cut off value of 10 kDa. Concentrated enzyme samples were mixed with equal volumes of glycerol and stored at −20 °C.

SDS-PAGE

SDS-PAGE was carried out following the protocol described by Laemmli[33] using 12.5% separation gels. The premixed protein standard "low range" (Roche Diagnostics, Mannheim, Germany) was employed as a marker. Silver staining was carried out according to Blum et al.[34]

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