Development of a Novel Biocatalyst for the Resolution of *rac*-Pantolactone

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Abstract: A novel L-pantolactone hydrolase, Lph, from *Agrobacterium tumefaciens* Lu681 was characterized, which stereospecifically hydrolyses L-pantolactone to L-pantoic acid yielding D-pantolactone with > 95% enantiomeric excess. The enzyme was found to be a 30 kDa-Zn^{2+}-hydrolase with a K_m for L-pantolactone of 7 mM and a V_{max} of 30 U/mg. The corresponding *lph* gene was identified as an 807 bp ORF and cloned into *E. coli*. It was overexpressed under control of P_{tac} and P_{rha} yielding enzyme activities of up to 600 U/g dry weight. Resolution of D,L-pantolactone in repeated batches with isolated Lph and enzyme recovery by membrane filtration gave D-pantolactone with 50% yield and 90–95% ee over 6 days. Covalent immobilization to EupergitC led to a stable biocatalyst easy to handle in a repeated batch production of D-pantolactone. Further improvements in the activity of Lph were achieved by directed evolution of the enzyme. Activities of mutants F62S, K197D and F100L were increased 2.3, 1.7, and 1.5 fold, respectively.

Keywords: enzyme catalysis; kinetic resolution; lactonohydrolase; D-pantothenic acid; Zn^{2+} hydrolase

Introduction

D-Pantolactone is an important intermediate in the production of D-pantothenic acid, also called vitamin B5. It is used mainly in feed for chicken and pigs and also as vitamin supply in human nutrition. While D-pantothenic acid has been traditionally produced by chemical processes which involve an efficient but troublesome diastereomer crystallization step, new biotechnical preparation methods have shown up in the literature during the last years.[1] Apart from microbial synthesis of D-pantothenic acid, especially the kinetic resolution of pantolactone by enantioselective enzymatic hydrolysis is an attractive approach since it not only fits into the old chemistry of D-pantothenic acid synthesis but also gives access to a second, related product, D-pantothenol. Today, the aldonolactonase of the fungus *Fusarium oxysporum* AKU3702 which hydrolyses D-pantolactone to D-pantoic acid[2] is used by Fuji/Daiichi Chemicals for the production of D-pantothenic acid on a multithousand-ton scale.[3] Recently, new pantolactone hydrolyzing enzymes with opposite enantioselectivity were purified from bacteria[4] (for a review of the diversity of lactonases see[5]).

Considering a kinetic resolution process, use of both a D- or an L-specific pantolactone hydrolase is possible in principle (Figure 1). Hydrolyzing the unwanted L-form, it may take longer to reach a sufficient enantiomeric excess for the remaining D-pantolactone, however, the process is much more robust, e.g., towards the competing spontaneous chemical hydrolysis. This paper describes the characterization and cloning of L-pantolactone hydrolase (Lph) from *Agrobacterium tumefaciens* Lu681 and its use for kinetic resolution of D-pantolactone. To our knowledge this is also the first report on directed evolution of a lactonase and it reveals the characteristics of some Lph mutants with increased L-pantolactone hydrolyzing activity.

Results and Discussion

Screening, Purification and Characterization of a Novel Enantioselective Pantolactone Hydrolase

After screening a variety of 965 bacterial and fungal strains, 17 strains were found to produce ≥ 1 mM L- or D-pantoic acid (ee > 70%) from D,L-pantolactone (50 mM) in 3 h, which did not belong to the known D-pantolactone hydrolyzing fungi. The L-selective pantolactone hydrolase from *Agrobacterium tumefaciens* Lu681 (formerly identified as *Burkholderia caryophylli*) was selected for further characterization (7.6 mM L-pantoic acid, ee 97%). The enzyme was purified as described in the experimental part and two peptides were identified after SDS-PAGE, trypsin digestion and Edman degradation, (1) YGIEGLNNLEAL and (2) AKEDANSTIEAED. Interestingly, the cysteine synthetases both from Lu681 and from recombinant *E. coli*

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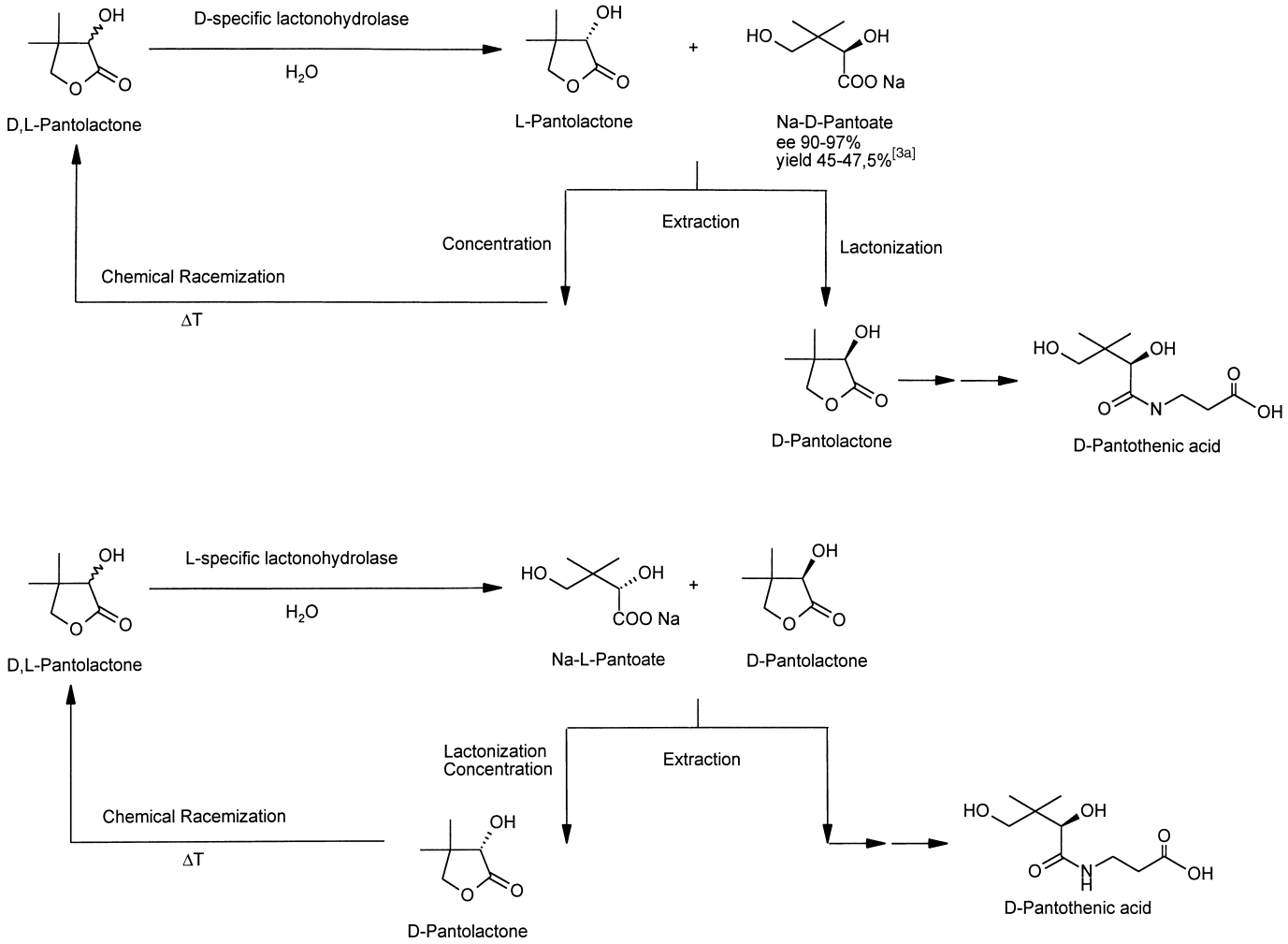

Figure 1. Production of D-pantothenic acid via resolution of D,L-pantolactone by either a D- or an L-specific lactonohydrolase.

were copurified by this method. Table 1 shows the substrate specificity of Lph. D-Pantolactone was not converted. Since there was no ester or lactone revealing a higher activity and L-pantolactone is not an obvious substrate in natural metabolism, it is not clear what the enzyme's physiological substrate might be. One idea is that it could be involved in the quorum sensing of the bacteria which typically proceeds *via* lactones. Although the enzyme showed neither inhibition by chelating agents (and a series of other enzyme inhibitors) nor significant activation by metal ions (Table 2) it turned out to be a Zn^{2+}-containing hydrolase by X-ray fluorescence spectroscopy of crystals. Renaturation of urea/EDTA (8 M/0.1 M)-denatured Lph in the presence of 1 μM Zn^{2+}, Mn^{2+}, Co^{2+} or Ni^{2+} restored 9.8, 6.0, 4.1 and 2.1% of the original activity (0.2% without addition of metal ions). The purified enzyme showed broad temperature and pH optima of 60–70 °C and 6.8–7.8, respectively. Since the competing chemical hydrolysis reaction increases at high pH and temperature, optical resolution of D,L-pantolactone was performed at pH 7.0–7.5 and 30 °C.[4a] The (apparent) K_m values were 10 mM and 7 mM for the purified enzymes from Lu681 and *E. coli*, respectively, and 33 mM for the whole cells of recombinant *E. coli*. V_{max} was about 30 U per mg protein.

Cloning and Expression of the L-Pantolactone Hydrolase from Lu681

For cloning of the Lph gene, a chromosomal library of Lu681 was constructed in *E. coli* XL1/pBluescriptSKII and screened for Lph activity by colony lift assay. One isolated positive clone carried a 7.5 kB insert with an ORF of 807 bp/269 aa (sequence in Figure 2) which contained the peptides 1 and 2 previously identified by Edman degradation. The homologies to public protein

Table 1. Substrate specificity of Lph from *Agrobacterium tumefaciens* Lu681.

Substrate	Concentration (mM)	Activity[a] [%]
L-Pantolactone	150	100
D-Pantolactone	150	>0.1
γ-Butyrolactone	150	13
γ-Valerolactone	150	4
δ-Valerolactone	50	2
ε-Caprolactone	150	32
(+/−)-δ-Decanolactone	15	n.d.[b]
δ-Nonalactone	75	11
(+)-Ethyl D-lactate	150	12
(−)-Ethyl L-lactate	150	40
D-Galactono-γ-lactone	150	10
L-Galactono-γ-lactone	150	57
L-(+)-Gulono-γ-lactone	150	9
D-(−)-Gulono-γ-lactone	150	9
1,2-*O*-Dilauryl-*rac*-glyceryl-3-glutaryl resorufin ester	0.25	1
5-Hydroxy-2-coumaranone	2.5	41
α-Naphthyl acetate	2.5	2
Isatine	10	7

[a] Activity for L-pantolactone was set to 100%.
[b] Although a decrease of (+/−)-δ-decanolactone indicated an activity equivalent to 75%, no (+/−)-δ-decanoic acid could be detected.

Table 2. Effects of various additives on the activity of Lph from *Agrobacterium tumefaciens* Lu681.

Substance	Concentration [mM]	Relative Activity [%]
No addition	–	100[a]
EDTA	1	97
Citrate pH 6.4	30	97
o-Phenanthroline	1	95
$HgCl_2$	1	133
p-Chloromercuribiphenyl-sulfonic acid	1	108
DTT	1	99
Phenylmethanesulfonyl fluoride	1	113
Diisopropyl fluorophosphate	1	115
Pepstatine	1	117
H_2O_2	1%	95
KCN	1	102
KCl	1	99
NH_4Cl	1	100
$MgCl_2$	1	99
$CaCl_2$	1	101
$MnCl_2$	1	100
$CoCl_2$	1	97
$FeCl_2$	1	104
$NiCl_2$	1	96
$ZnCl_2$	1	113
SDS	1%	102
CHAPS	0.1%	104
Tritone	0.1%	104
2-Propanol	10%	93
Acetonitrile	10%	119
Methanol	10%	100

[a] Activity was set to 100%.

sequences were low except for hypothetical proteins from *Agrobacterium tumefaciens*, *Sinorhizobium meliloti* and *Nostoc* sp. (see Table 3). However, although isatine is a bad substrate for Lph, isatine hydrolase[6] is the fully characterized protein most closely related to Lph. Peptide sequences of the lactonohydrolase from *Agrobacterium tumefaciens* AKU316 identified so far differ only in 10 from 66 residues characterized by Edman degradation. Despite the striking similarity, this enzyme was described to have a higher pH optimum of 8.0–8.5[4b] than Lph from *Agrobacterium tumefaciens* Lu681. Since the spontaneous hydrolysis rate of pantolactone increases with higher pH, better ee values are obtained in bioconversions around pH 7.0.

The *lph* gene was cloned into pKK223-3- and pDHE19.2[4a,7]-expression vectors for IPTG- and rhamnose-dependent overexpression in *E. coli*, respectively (resulting plasmids: pKK681 and pDHE681). The portion of soluble and active Lph was increased to approximately 10% of total protein by coexpression of *groELS* (Figure 3). Up to 600 U/g (cell dry weight) were produced in a 12 L batch fermentation compared to 150 U/g without *groELS* and 3 U/g in Lu681. For convenient production of the biocatalyst with the pDHE system, a rhamnose-deficient *E. coli* strain can be used.

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     M  C  N  N  C  V  I  E  N  V  K  K  N  M  L  S  R  R  L  L
1    ATGTGCAACA ACTGCGTGAT CGAGAACGTA AAAAAGAACA TGCTTTCACG GCGCCTGCTG
     F  K  G  A  A  A  G  L  T  A  M  T  A  G  S  L  A  S  P  A
61   TTCAAGGGCG CTGCGGCAGG TTTGACGGCC ATGACGGCAG GCAGTCTGGC TTCCCCGGCG
     L  A  Q  S  P  R  Q  V  V  D  L  T  H  T  Y  D  S  A  F  P
121  CTTGCGCAAT CGCCCCGGCA GGTCGTTGAT CTCACTCACA CCTATGATTC CGCATTTCCC
     T  F  D  G  K  P  G  I  E  Y  E  W  A  A  Q  I  A  K  D  G
181  ACCTTCGATG GCAAACCGGG CATAGAATAT GAGTGGGCAG CGCAGATCGC CAAAGACGGC
     Y  Q  L  R  K  L  T  I  Y  E  H  T  G  T  H  I  D  A  P  F
241  TATCAGCTCC GCAAACTCAC CATCTACGAA CATACCGGCA CCCATATCGA TGCGCCTTTC
     H  F  S  A  D  G  A  S  V  D  Q  L  E  P  Q  K  L  V  A  P
301  CACTTCAGCG CCGATGGCGC GAGCGTCGAC CAACTGGAGC CGCAGAAACT TGTCGCTCCG
     L  V  I  V  D  I  T  E  R  A  K  E  D  A  N  S  T  I  E  A
361  CTTGTCATCG TCGACATCAC CGAGCGCGCC AAAGAGGATG CCAATTCCAC CATTGAAGCC
     E  D  I  E  R  W  I  S  A  N  G  D  I  P  T  G  A  I  V  A
421  GAAGACATCG AGCGCTGGAT ATCTGCGAAT GGCGACATCC CGACAGGTGC AATCGTGGCT
     L  R  S  G  W  A  T  K  V  K  S  P  S  F  R  N  D  E  A  G
481  TTACGCTCCG GATGGGCAAC CAAAGTGAAG AGTCCCTCAT TCCGCAATGA CGAAGCCGGA
     Q  F  A  F  P  G  F  G  K  S  A  T  D  L  L  L  K  L  D  T
541  CAATTCGCCT TCCCCGGTTT CGGCAAATCG GCGACCGACC TTCTGCTGAA GCTCGACACC
     V  A  I  G  V  D  T  L  S  L  D  P  G  N  S  A  D  F  A  V
601  GTCGCCATTG GCGTCGACAC ACTTTCTCTG GATCCGGGCA ACTCCGCAGA TTTCGCGGTT
     H  N  S  W  L  P  A  G  R  Y  G  I  E  G  L  N  N  L  E  A
661  CACAATTCCT GGCTGCCAGC AGGACGCTAC GGTATCGAAG GACTGAACAA CCTCGAGGCT
     L  P  V  K  G  A  T  I  I  V  G  A  P  A  H  R  G  G  T  G
721  CTGCCGGTCA AGGGAGCGAC CATAATCGTC GGCGCGCCGG CACACCGCGG CGGAACGGGC
     G  P  A  R  I  L  A  L  V
781  GGCCCAGCCC GTATTCTGGC CCTGGTC
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Figure 2. DNA sequence of the *lph* gene and deduced peptide sequence of Lph. Alignment with homologous sequences (Table 3) reveals a very conserved region (consensus sequence htgTHiDAPxHf, bold letters). Peptides identified by Edman degradation of purified Lph are shown in italic letters.

Table 3. Homologies of Lph.

Homologous Sequence	Accession No. (or Reference)	Organism	Homology[8] (% aa)	Identity[8] (% aa)
AGR_L_227p	AAK88681	*Agrobacterium tumefaciens*	93.3	90.3
Hypothetical transmembrane protein	NP 386248	*Sinorhizobium meliloti*	59.3	52.2
Hypothetical protein	NP 487809	*Nostoc* sp.	46.6	38.6
Isatine hydrolase	WO9119175	*Pseudomonas putida*	47.9	37.4
Hypothetical protein	NP 519956	*Ralstonia solanacearum*	43.8	34.3
Hypothetical protein	B69206	*M. thermoautotrophicum*	42.6	29.8
Hypothetical protein	D70817	*M. tuberculosis*	37.1	28.5
Hypothetical protein	B72430	*T. maritima*	45.4	31.9
Hypothetical protein	G69399	*Archaeoglobus fulgidus*	35.5	26.3
Hypothetical protein	S39964	*S. griseus*	39.6	29.8
Hypothetical protein	S75497	*Synechocystis* sp.	36.3	26.8
Hypothetical protein	T06135	*A. thaliana*	31.3	23.1
Hypothetical protein	T05418	*A. thaliana*	33.0	23.7
Polyketide synthase	O68500	*S. peuceticus*	42.7	32.6
Tetracyclin synthase	O86485	*S. argillaceus*	43.2	34.9
ORF	Q54196	*S. griseus*	39.6	29.8

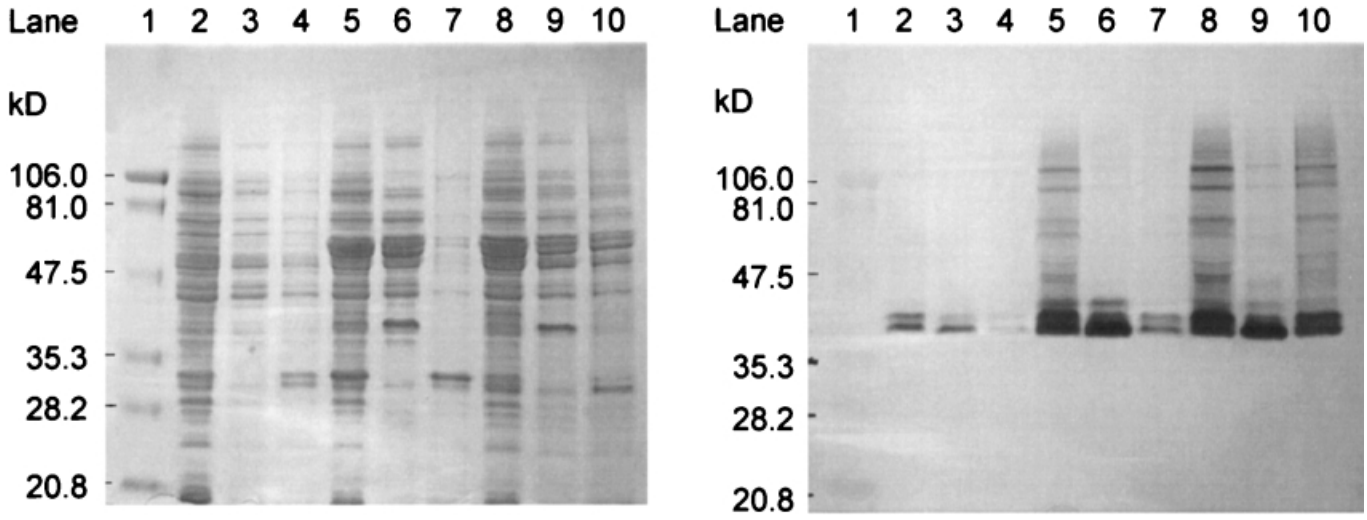

Figure 3. Expression of *lph* under control of P_{Rha} in *E. coli* with (Lu9981) and without (Lu9871) coexpression of *groELS*. Coomassie- (left) and activity-staining with α-naphthyl acetate and Fast-Red (right). MW-marker (1), Lu9871 cells (37 °C), whole fraction (2), Lu9781 supermatant (3) and Lu9871 pellet (4), (5) – (7) Lu9981 (37 °C) analogues, (8) – (10) Lu9981 (30 °C) analogues. Lane 6 indicates the increase of soluble and active Lph with coexpression of *groELS* compared to lane 3. Growth at 30 °C does not show further advantage.

Resolution of Pantolactone with Lph Extract

For an economic resolution process of *rac*-pantolactone, the repeated use of the corresponding catalyst is required. *E. coli* cells suspended in a reactor vigorously stirred for quick titration of the synthesized pantoic acid and containing high concentrations of pantolactone and Na or NH_4 pantoate tend to lyse and are hard to be recovered from the product solution. Therefore, Lph was used as isolated enzyme after heat precipitation and membrane filtration of the *E. coli* extract. Repeated conversion of D,L-pantolactone (30% w/v) with this homogenate gave D-pantolactone in 50 – 53% yield with 90 – 95% ee for 6 batches (6 d; Figure 4). Lph and the product solution were separated by membrane filtration for each batch. Taking into account that 8% of solution was lost during each filtration step due to the residual volume of the laboratory equipment, a half-life-time of 12 (instead of 6) days might be estimated. The reason for the decrease in reaction velocity for each conversion was found to be a competitive inhibition by D-pantolactone (Eq. 1) and a slight product inhibition of Lph. Under the same conditions of pH and temperature, L-pantolactone was completely converted to L-pantoic acid.

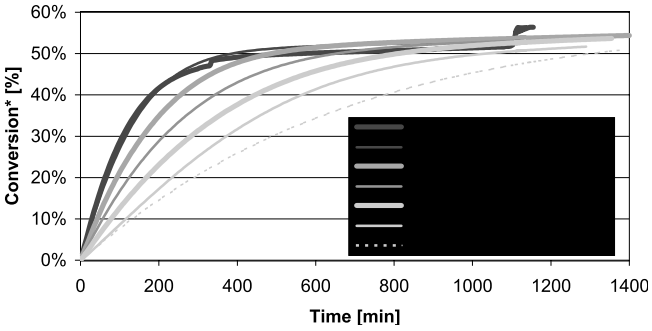

Figure 4. Resolution of pantolactone with Lph extract, repeated batch assay (7 days). The reaction process was followed by titration of the produced pantoic acid by 4 M NaOH. *: conversion according to NaOH consumption. Conversions given in the inset were determined by HPLC analysis.

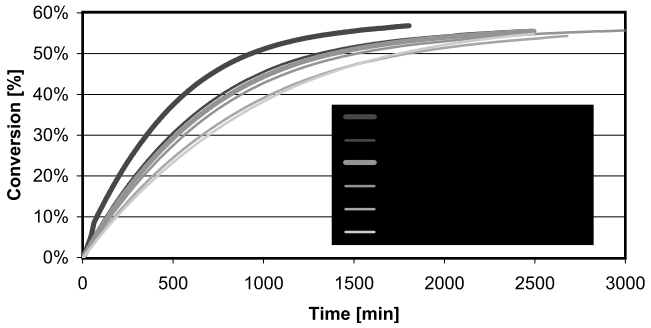

Figure 5. Resolution of pantolactone with EupergitC-Lph, repeated batch assay (6 days). The reaction process was followed by titration of the produced pantoic acid by 10 M NaOH. *: conversion according to NaOH consumption. Conversions given in the inset were determined by HPLC analysis.

After filtration of a homogenate resolution mixture with > 50% conversion and > 90% ee, D-pantolactone can be extracted with ketones or esters of medium polarity with good yields.[9] L-Pantoic acid can be recycled from the aqueous layer by addition of mineral acid and heating as indicated in Figure 1.

$$\frac{1}{V} = \frac{1}{V\max} + \frac{Km}{V\max}\left(1 + \frac{[D-PL]}{K_{D-PL}}\right)\frac{1}{[L-PL]} \qquad (1)\,[10]$$

Immobilization of Lph and Resolution of Pantolactone with Immobilized Lph

From several attempts to efficiently and cheaply immobilize Lph, covalent attachment to EupergitC (Röhm GmbH, Darmstadt, Germany) worked best. Binding of Lph from homogenate to EupergitC proceeded with 55 – 70% yield achieving specific activities of 24 – 81 U/g (wet weight). *E. coli* cells could easily be entrapped into various forms of alginate beads with good yield of activity, however, the performance of these immobilizates in the repeated batch reaction was poor in terms of mechanical stability.

The repeated batch conversions of D,L-pantolactone with Eupergit-Lph show kinetic characteristics similar to those of the reactions with free enzyme (Figure 5). Depending on the solid portion (> 8 – 10%), the stirring geometry and velocity, Eupergit-Lph immobilizate was slowly ground to fine particles that tended to clog the filters during product recovery. Nevertheless, even with this unoptimized system the half-life-time of Lph was extended to 13 days.

Directed Evolution of Lph

For reduction of biocatalyst costs in the production process of D-pantolactone, an improved specific activity of Lph was desirable. A library of 11680 *lph* mutants was prepared by error prone PCR with a mutation rate of 1.9 bp/*lph* gene (1.36 aa/Lph-variant) and a transition to transversion rate of 0.89 (corresponding to 53% active clones). After screening for increased L-pantolactone hydrolyzing activity, 3 isolated mutants F62S, K197D and F100L with 2.3, 1.7 and 1.5 fold activity in the screening assay were verified and characterized so far (Table 4).

While mutant F62S shows excellent activity in the standard enzyme assay, its performance under process conditions (high concentrations of D,L-pantolactone) was poor. Since close adaptation of the high throughput screening assay to these conditions is very difficult this example shows the challenge of assay development for improvement of *industrially valuable* catalysts. It also shows that the factors of improvement achievable by only one round of random mutagenesis and screening strongly depend on what the starting point is, because obviously, like in this case, the specific activity of an already good biocatalyst is not easily improved by

Table 4. Comparison of Lph mutants.

Mutation	Number of Clones	Specific Activity[a]	Specific Activity with 30% D,L-Pantolactone[b]
F62S	4	227%	> 70%
K197D	2	171%	120%
F100L	3	149%	120%

[a] Activity according to the standard enzyme assay with 0.15 M L-pantolactone compared to the activity of the wild-type clone with *groELS* coexpression (TG1 pKK681 pAgro4 pHSG575).

[b] Resolution of D,L-pantolactone was performed as described below. 50 – 54% conversion and ee > 90% were reached after addition of only 80% of biomass compared to the wild-type clone with groELS coexpression (TG1 pKK681 pAgro4 pHSG575) under the same conditions.

factors in the range of 10–100 like in cases where the starting point was substantially lower.[11] Resolution of D,L-pantolactone by mutants K197D and F100L gave D-pantolactone with 90.4% ee at 51.8% conversion and 90.2% ee at 50.1% conversion, respectively, after 720 min using only 80% of the biomass compared to the unmutated control (90.5% ee at 52.3% conversion after 900 min). This corresponds to an increase of the productivity of the recombinant biocatalyst from 136 to 170 g/g (g D-pantolactone per g biomass) and a slight shortening of the reaction time down to 12 h.

Conclusion

The results presented here are promising for industrial utilization of recombinant Lph from *Agrobacterium tumefaciens* Lu681 for the resolution of D,L-pantolactone in a process for the production of D-pantothenic acid. Further progress might be obtained by saturation mutagenesis of the aa positions 62, 100 and 197, by combining the mutations and introducing these mutants into further evolutionary cycles or a shuffling procedure.[12] Future investigations on the crystal structure of Lph are expected to reveal molecular details of the enzymatic L-pantolactone hydrolysis. Structural information about Lph might also be used to design an enzyme not inhibited by excesses of D-pantolactone.

Experimental Section

Strains and Chemicals

Agrobacterium tumefaciens Lu681 (DSMZ 13050, formerly identified as Burkholderia caryophylli) was grown overnight in Luria Bertani medium in flask cultures or a 12 L fermenter at 30 °C. All chemicals were purchased from Sigma-Aldrich Chemie GmbH, Munich, unless otherwise stated. Sodium pantoate was synthesized by complete hydrolysis of pantolactone: 20 μL of 12 M NaOH were added to 1 mL of 100 mM pantolactone and heated for 5 min at 95 °C. The solution was neutralized by addition of 13 μL of concentrated HCl and dilution in 50 mM Tris/HCl buffer, pH 7.0.

Enzyme Assay

L-Pantolactone hydrolase activity was routinely assayed for 1 h at 30 °C in a mixture containing 150 mM Pipes pH 7.0, 150 mM L-pantolactone and an appropriate amount of enzyme or cells (0.1 U/mL). The reaction was stopped on ice and the cells/enzyme filtered off using a 10 kDa membrane spin filter. Filtrates were analyzed by HPLC using a 2.1 mm × 150 mm Alltima C18-column (5 μm, Alltech GmbH, Unterhaching, Germany) and a linear gradient of 5–50% acetonitrile in potassium phosphate (100 mM, pH 2.0) in 6 minutes at 0.3 mL/min on an HP1090 (oven temperature 65 °C) with diode-array detection. The pantoic acid and pantolactone signals were monitored at 220 nm. D- and L-pantoic acid were determined by chiral HPLC analysis of the filtrates as described previously[13] with modified flow (0.7 mL/min) and slightly increased acetonitrile concentration (8%).

Purification of L-Pantolactone Hydrolase from Lu681 and Activity Staining after SDS-PAGE

1128 g of wet pellet were vigorously resuspended in 1.8 L 20 mM Tris/HCl, pH 7.4, buffer to an end volume of 3 L. This solution was freed of small particles by filtration on a bed of glass beads (0.1–0.2 mm). Thereafter, the cells were homogenized in a microfluidizer (1000 bar). After washing of the system the total volume was 4 L.

To 4 L homogenate 200 mL 1 M $MnCl_2$ were added. The pH was continuously adjusted to pH 7.0. The precipitate was removed by centrifugation. To the cleared solution we added 200 mL of a 0.2 M EDTA solution (pH 7.5) and the pH dropped to 5.0. The newly formed precipitate was also removed by centrifugation and the pH was readjusted to 7.0. Then, more protein was precipitated by ammonium sulfate (50% saturation). The precipitate was removed by centrifugation and the supernatant (3.7 L) was divided in 1.2 L fractions.

For hydrophobic chromatography one 1.2 L fraction was applied to a phenyl-sepharose column (Pharmacia, 5 cm diameter, 25 cm height, 490 mL volume). The column was washed with 1 L buffer A (20 mM sodium phosphate, pH 7.4, 40% ammonium sulfate). Active fractions were eluted in a linear gradient to 100% buffer B (20 mM sodium phosphate, pH 7.4; 120 min, 10 mL/min) and collected (250 mL). They were diluted to a conductance of less than 7 ms/cm (3 L) and applied to a Q-sepharose column (Pharmacia Fast Flow, 5 cm diameter, 25 cm height, 490 mL volume) for ion exchange chromatography. The column was washed with 1 L buffer B (10 mL/min) and eluted with buffer C (buffer B with 1 M NaCl) in a linear gradient during 2 h to 100% buffer C. Active fractions were collected (118 mL) and concentrated by ultrafiltration (10 kDa, Omega Membrane) and then dialysed against 10 mM Tris/HCl pH 7.0 (21 mL). 6 mL of this preparation were applied to a Waters Q HR8. The column was equilibrated with 20 mM MES, pH 6.0. The enzymatic activity was eluted with the same buffer including 500 mM of NaCl in a linear gradient at a velocity of 1% per minute to 100%. Active fractions were dialysed and centrifuged and then applied to Mono-P chromatography (Pharmacia, 0.5 cm diameter, 5 mL). The enzymatic activity was again eluted with NaCl and the active Mono-P fractions were precipitated with cold acetone and applied to non-reducing SDS-PAGE without heating the sample (12% Tris/glycine minigels, 2.5 h, 125 V, 50 mA; 0.4% SDS in the sample buffer).

After SDS-PAGE the gel was briefly washed in Tris buffered saline and incubated in 50 mL TBS with α-naphthyl acetate (Sigma N8508, 20 mg in 5 mL acetone + 45 mL water) for 10 min. After washing the gel was incubated with 50 mL Fast Red solution (Sigma F-8764, 50 mg in 50 mL water). Esterases (and Lph) form red bands. Coomassie stained bands in a second set of gels were cut out and digested in the gel slices with trypsin. Peptides were isolated by C18 HPLC and sequenced by Edman degradation.

Cloning and Sequencing of the L-Pantolactone Hydrolase Gene

Genomic DNA of Agrobacterium tumefaciens Lu681 was isolated with QIAGEN Genomic Tips, partially digested with EcoRI and cloned into pBluescriptIISK (Stratagene). After transformation into E. coli XL1Blue ultracompetent cells (Stratagene), colonies were replicated on nitrocellulose filter (HAWP09000, Millipore) and screened for activity: The filters were sequentially soaked in 1% SDS/10 mM Tris/HCl pH 7.0 (5 minutes at room temperature), 10 mM Tris/HCl pH 7.0 (5 minutes at room temperature) and 150 mM L-pantolactone/ 0.1% nitrazine yellow/10 mM Tris/HCl pH 7.0 (from 3 minutes up to 15 h at 30 °C). Positive colonies turned yellow and were further characterized by DNA sequencing, subcloning into expression vectors pKK223-3 (Pharmacia) and pDHE19.2 (a derivative of pJOE2702, Stuttgart[4a,7]) and biochemical characterization.

Growth of Recombinant *E. coli* and Preparation of L-Pantolactone Hydrolase Homogenate

For cultivation in flasks or microplates E. coli strains were grown in Luria Bertani medium with appropriate antibiotics and inducing agents at 37 °C (ampicillin 100 μg/L, spectinomycin 50 μg/L, chloramphenicol 10 μg/L, IPTG 0.1 – 1 mM, rhamnose · H_2O 2.5 g/L).

200 mL of rich tryptone-yeast medium with 100 μg/mL ampicillin, e.g., 13.3 g/L KH_2PO_4, 4.0 $(NH_4)_2HPO_4$, 1.2 g/L $MgSO_4 \cdot 7\,H_2O$, 0.2 g/L $CaCl_2$, 1.7 g/L citric acid, 15.0 g/L tryptone (Difco), 66.6 g/L yeast lysate 65% (Fould Springer), 10 mL/L trace metal solution 100,[14] 0.1 g/L Fe(III) citrate, pH 7.0 with NaOH, were inoculated with *E. coli* TG1 pDHE681 or TG1 pDHE681 pAgro4 pHSH575 and grown for 8 h at 37 °C and 200 rpm. The preculture was added to 10 L of glycine-tryptone-yeast medium (13.3 g/L KH_2PO_4, 4.0 $(NH_4)_2HPO_4$, 1.2 g/L $MgSO_4 \cdot 7\,H_2O$, 1.7 g/L citric acid, 15.0 g/L tryptone (Difco), 5 g/L yeast lysate 65% (Fould Springer), 40 g/L glycerine 99.5% (DAB13, Riedel Haën), 10 mL/L trace metal solution 100,[14] 0.1 g/L Fe(III) citrate, 0.1 g/L Tego KS911 antifoam). Culturing conditions were 37 °C, pH 7.0 (25% NaOH), ≥ 600 rpm ($pO_2 \geq 25\%$). For TG1 pDHE681 pAgro4 pHSH575 cultivation, 0.15 mM IPTG was added after 1 h for induction of chaperone expression. For induction of Lph expression, rhamnose (2.5 g/L rhamnose · H_2O) was added after 1 h 15 min and kept at 1.0 to 2.0 g/L in concentration by automatic HPLC control and addition of a 10% solution. For rhamnose-deficient E. coli strains, adding once 0.5 g/L rhamnose is sufficient. After 8 h cells were harvested for whole cell assays (typical yields were 600 U/g dry weight and 8000 – 10000 U/L) or immediately disrupted in a microfluidizer (2 × 1000 bar) at 4 – 10 °C. After centrifugation (20 min at 9000 g, 10 °C) Lph was concentrated (factor 10) by cross-flow filtration (Hämoflow F60, Fresenius, 10 kDa membrane). Heat precipitation of other proteins (20 min 60 °C; 20 min 10 °C, centrifugation as above) led to a homogenate reaching ca. 3000 U/g protein and 90000 U/L (> 90% yield of activity and 49% protein content compared to the starting cell material). The homogenate was stable for 2 d at 4 °C and > 3 months at − 20 °C.

Immobilization of L-Pantolactone Hydrolase

To 300 mL homogenate 17.5 g NaCl were added and the pH was adjusted to 6.8. After addition of 15 g dry EupergitC (Röhm AG, Darmstadt) the suspension was incubated for 17 h at room temperature. The Lph-EupergitC immobilizate (60 g wet wt) was washed with water and stored in 10 mM phosphate buffer pH 7.5.

Resolution of D,L-Pantolactone in a Repeated Batch Process

A 20 – 1000 mL mixture of D,L-pantolactone (300 g/L = 2.3 M), 10 mM $NaHCO_3$ (optional) and either Lph homogenate (16000 U/L) or immobilized Lph (16000 U/L or max. 80 g/L) was stirred at 30 °C with automatic control of the pH by titration with 4 or 10 M NaOH (pH 7.5). After complete hydrolysis of L-pantolactone (D-pantolactone ee ≥ 90%, yield ≥ 50%), the enzyme was recovered by membrane filtration (homogenate, see above) or by aspiration through an HPLC-filter (solvent filter 655-1395, Merck), washed twice with 1 volume of water and used again.

Generation of Lph Mutants

Lph mutants were generated by error-prone PCR with Taq-polymerase (2.5 U, Roche) and pKK681 as template (2 ng/μL using the primers 5′-CCGGAATTCATGTGCAACAAC-TGC and 5′-CCCAAGCTTCAGACCAGGGCCAGAA. Other concentrations were 40 μM ATP and GTP each, 200 μM CTP and TTP each, 0.5 mM $MgCl_2$, 1 M GC rich resolution solution (Roche, contains DMSO), GC rich reaction buffer (1x, Roche). The PCR program consisted of 3 min 95 °C, 10 min 85 °C, 4 cycles of 0.5 min at 54 °C, 2 min at 72 °C, 0.5 min at 95 °C and 26 cycles of 0.5 min at 58 °C, 2 min at 72 °C, 0.5 min at 95 °C and finally 10 min at 72 °C and cooling to 4 °C. PCR products were isolated by E-Gel electrophoresis (Invitrogen) and elution with Qiaquick or GFX-Kit (Qiagen or Pharmacia, respectively). The isolated PCR products were cloned with EcoRI/HindIII into pKK223-3. Ligation mixtures were transformed into XL1 Blue competent cells containing pAgro4 and pHSG575 for chaperone coexpression. Colonies were transferred into 96-well microplates for screening. Controls were prepared by standard PCR (Pfu polymerase) and transferred into each microplate (A1-D1).

High Throughput Assay for Screening of Lph (and Other Acid Producing Enzymes)

A preculture of the mutants was grown in microplates ($LB_{amp\ spec\ cm}$) for 18 h at 37 °C with normal shaking at 220 rpm and used for inoculation of a similar, induced culture containing 0.15 mM IPTG. After growth (18 h at 37 °C) the cells were evenly suspended and 20 μL automatically diluted into 80 μL 10 mM Tris/HCl pH 7.0. Growth was checked by measurement of optical density at 600 nm. To start the assay 100 μL of 0.6 M rac-pantolactone/0.01% nitrazine yellow pH 4.3 were added and decrease of the extinction was measured at 590 nm every 10 min for 1 h. Clones were evaluated by their decrease coefficient determined by linear

regression ("activity"). Since the standard deviation of positive controls was 21.5%, only clones showing > 120% activity compared to the control level were reassayed. Clones showing again > 120% activity were precultured overnight and grown in 600 mL cultures for 6 h at 37 °C. Cells were harvested and tested by an enzyme standard assay. Under these conditions the wild-type clone reached 1200 U/L. Different amounts of biomass were used for the resolution of 30% (w/v) D,L-pantolactone in comparison with resolution batches (20 mL, see above) with the wild-type clone. The latter gave 52.3% conversion and ee 90.5% in 15 h with addition of a cell suspension equivalent of 10.5 g/L dry biomass (=100%; 13000 U/L).

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