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A stereoselective carbon-nitrogen lyase from *Ralstonia* sp. SLRS7 cleaves two of three isomers of iminodisuccinate

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Abstract

Following biodegradation tests according to the OECD guidelines for testing of chemicals 301F different degradation rates were observed for the three stereoisomers of iminodisuccinate (IDS). A strain was isolated from activated sludge, which used two of three isomers, *R*,*S*-IDS and *S*,*S*-IDS, as sole source of carbon, nitrogen, and energy. The isolated strain was identified by 16S-rDNA and referred to as *Ralstonia* sp. SLRS7. An IDS-degrading lyase was isolated from the cell-free extract. The enzyme was purified by three chromatographic steps, which included anion-exchange chromatography, hydrophobic interaction chromatography and gel filtration. The lyase catalysed the non-hydrolytic cleavage of IDS without requirement of any cofactors. Cleavage of *S*,*S*-IDS led to the formation of fumaric acid and L-aspartic acid. Interestingly *R*,*S*-IDS yielded only D-aspartic acid besides fumaric acid. *R*,*R*-IDS was not transformed. Thus, the IDS-degrading enzyme is a carbon–nitrogen lyase attacking only the asymmetric carbon atom exhibiting the *S*-configuration. Besides *S*,*S*-IDS and *R*,*S*-IDS cleavage, the lyase catalysed also the transformation of certain *S*,*S*-IDS metal complexes, namely Ca^{2+}-, Mg^{2+}- and Mn^{2+}-IDS. The maximum enzyme activity was found at pH 8.0–8.5 and 35 °C. SDS-PAGE analysis revealed a single 57-kDa protein band. The native enzyme was estimated to be around 240 kDa indicating a homotetramer enzyme.

Abbreviations: EDDS – ethylenediaminedisuccinate; EDTA – ethylenediaminetetraacetate; IDS – iminodisuccinate; NTA – nitrilotriacetate

Introduction

Metal chelators, also known as sequestrants or metal binding agents, form strong water-soluble complexes with most alkaline earth and heavy-metal ions. These chelators are used for a wide variety of applications, e.g., to stabilise the bleaching process in the textile and the pulp and paper industry, to control water hardness ions that interfere with cleaning products, to make micro nutrients bioavailable for agriculture, and in photographic film processing, to name only a few. The two most widely used synthetic chelating agents ethylenediaminetetraacetate (EDTA) and nitrilotriacetate (NTA) belong to the group of aminopolycarboxylates (Figure 1). NTA is readily biodegradable (Siegrist et al. 1988; Alder et al. 1990). A summary of NTA-degrading bacteria isolated from various habitats was given in a review by Bucheli-Witschel & Egli (2001). The more effective chelator EDTA is poorly biodegradable in municipal sewage treatment plants (Siegrist et al. 1988; Alder et al. 1990; Kari & Giger 1996) despite some encouraging results with selected axenic cultures (Lauff et al. 1990; Witschel et al. 1997; Nörtemann 1999). Photochemical degradation of

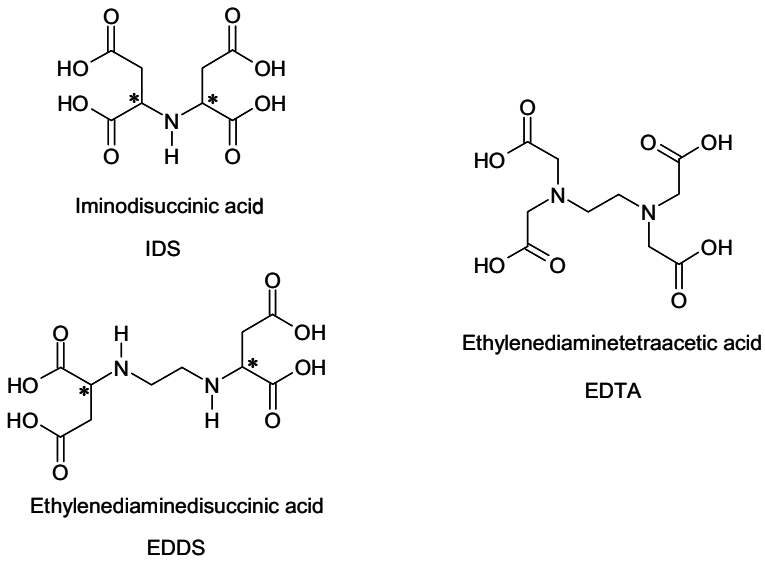

Figure 1. Structural formulas of the chelating agents IDS, EDDS, and EDTA. Asymmetric carbon atoms are marked with asterisk.

Fe^{3+}-EDTA in surface waters is presently the only known natural attenuation (Kari et al. 1995).

EDTA is the most commonly used metal chelator, because of its advantageous chemical properties. As a result, EDTA is released into the environment, particularly to surface waters. Ubiquitous distribution of EDTA may have some undesirable consequences such as the remobilisation of heavy-metals and radionuclides from soils, sediments and infiltration areas (Gardiner 1976; Means et al. 1978; Means & Alexander 1981). Theoretically, the mobilised toxic heavy-metals and radionuclides can be accumulated by plants and transferred to human beings through the food chain or can cause problems in the preparation of drinking water. Accordingly, there is an increasing interest to replace the persistent EDTA by an equally effective but readily biodegradable chelating agent.

Promising aminopolycarboxylates as alternative chelating agents are ethylene diaminedisuccinate (EDDS) and iminodisuccinate (IDS). The chemical structures are shown in Figure 1. Importantly these structures harbour two asymmetric carbon atoms generating three different stereoisomers (*R*,*R*; *R*,*S*; *S*,*S*). In the case of EDDS, the *S*,*S*-EDDS isomer is readily biodegradable whereas the *R*,*R*-EDDS persists and *R*,*S*-EDDS leads to the formation of a recalcitrant end product (Schowanek et al. 1997). In the light of these results it is notable that up to now only the *S*,*S*-EDDS isomer was found to be produced naturally by *actinomycetes* (Nishikiori et al. 1984).

IDS is to be classified as a synthetic, medium strength chelating agent and can be synthesised from maleic anhydride, ammonia and sodium hydroxide. This synthesis route produces a mixture of stereoisomers consisting of 25% *S*,*S*-IDS, 50% *R*,*S*-IDS, and 25% *R*,*R*-IDS. IDS is classified as readily biodegradable on the basis of two different standardised biodegradation tests. The OECD 302B test (Zahn–Wellens test) and the OECD 301E test (modified OECD-screening test) achieved a DOC-consumption of 89–99% and 79%, respectively after 28 days (Bayer 1998, 2001, 2002). Investigations on the metabolism of IDS by *Achromobacter xylosoxidans* B3 revealed liberation of aspartic acid but further details on the mechanism are missing (Reinecke et al. 2000). In this publication we report on the isolation of a stereoselective IDS-degrading strain and describe the stereoselective initial transformation of *R*,*S*-IDS and *S*,*S*-IDS.

Materials and methods

Characterisation of the IDS samples

Na_4-IDS isomer mixture (Baypure CX 100 solid): The amount of iminodisuccinate sodium salt was minimum 65% and the solid content (sum of

sodium salts) was minimum 85%. The sample contained 50% *R*,*S*-IDS, 25% *S*,*S*-IDS, and 25% *R*,*R*-IDS.

The isomeric purity of the *S*,*S*-IDS reference was 100%. The *R*,*R*-IDS isomer sample was 94.2% pure, by-products were 4.1% *R*,*S*-IDS and 1.7% fumaric acid. The *R*,*S*-IDS isomer sample was 94.9% pure, and contained 4% H_2O and 1.1% (*R*,*R*-IDS + *S*,*S*-IDS). *R*,*S*-IDS, *S*,*S*-IDS, *R*,*R*-IDS as well as the IDS isomer mixture were gifts from the Bayer AG (Leverkusen, Germany).

Chemicals

All other chemicals were of analytical grade and were purchased from Aldrich (Steinheim, Germany), Fluka (Neu-Ulm, Germany), Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany), or Sigma (Deisenhofen, Germany).

Biodegradation test

The biodegradation tests with IDS were performed as described in the OECD guideline for the testing of chemicals (manometric respirometry test 301F). The test was performed with the OxiTop® Control system (WTW, Weilheim, Germany) and inoculation was done with freshly collected activated sludge from the municipal sewage treatment plant in Stuttgart-Büsnau (University Stuttgart, Germany). Relative oxygen consumption was calculated on the basis of the chemical oxygen demand (COD). The COD was measured with the Dr. Lange test system LCK 314 (Düsseldorf, Germany) according to ISO 6060.

Bacterial strain and growth conditions

Ralstonia sp. SLRS7 was isolated from activated sludge of the municipal sewage treatment plant (Stuttgart-Büsnau, Germany) and identified by 16S-rDNA sequencing.

For isolation and growth of *R*. sp. SLRS7 and batch experiments, the following mineral salts medium without nitrogen was used: 20 mg/l Fe(III)-citrate, 1 g/l $MgSO_4 \times 7H_2O$, 50 mg/l $CaCl_2 \times 2H_2O$ and 1 ml trace element solution (Pfennig & Lippert 1966). The mineral salts medium was buffered with Na^+/K^+-phosphate (50 mM, pH 7.4) and supplemented with IDS (5–20 mM) as the sole source of carbon, nitrogen, and energy. The aqueous stock solution of the IDS isomer mixture (500 mM) was adjusted to pH 7 with hydrochloric acid. The free acids of the isomeric pure IDS samples were dissolved in water and adjusted to pH 7 with sodium hydroxide. The cells were grown in baffled Erlenmeyer flasks on a rotary shaker at 125 rpm and 23 °C. Cultivation was done on solidified media containing 1.5% (w/v) agar.

For the production of high biomass amounts for protein purification, the strain was grown in batch cultures in a 10 l aerated fermenter (BIO-MAG, BCC, Göttingen, Germany) at 23 °C, 400 rpm and 1.2 l air min^{-1}. The cells were harvested immediately after consumption of IDS at an optical density at 546 nm of 1.5–3 by centrifugation and washed once with 0.05 M Tris/HCl (pH 8.0). Cells were frozen in liquid nitrogen and stored at −30 °C. Transformation of different metal-IDS complexes by *R*. sp. SLRS7 was monitored by measuring concentrations of dissolved organic carbon (DOC) in cell-free culture fluids with a total organic carbon analyser (TOC-analyzer micro N/C, IDC, Langewiesen, Germany).

Preparation of cell extract

Fifteen grams (wet weight) of frozen cell paste was thawed at room temperature and resuspended in 40 ml of 0.05 M Tris/HCl (pH 8.0). The cells were broken by three passages through a French press (SLM Aminco, Urbane, Ill., USA) at 7 MPa. After the first passage 10 mg of DNase II (Serva, Heidelberg, Germany) was added. The suspension was kept on ice after each passage. The crude extract was centrifuged for 45 min at 100,000 × g to remove cells and cell debris. The resulting cell-free extract was used after filtration through 0.22 μm-pore-size filters for enzyme assays and protein purification.

Purification of the IDS-degrading enzyme

The cell-free extract was dialysed 18 h at 6 °C (dialysis tubing size 3, molecular weight cut off 12–14 kDa; Medicell Int. Ltd., London) against 0.05 M Tris/HCl (pH 8.0) and then loaded on an anion exchange column (Q-Sepharose HR, 1.6 by 10 cm; Pharmacia, Uppsala, Sweden) pre-equilibrated with 0.05 M Tris/HCl buffer (pH 8.0) at a flow rate of 2.0 ml min^{-1}. Fractions containing *S*,*S*-IDS-degrading activity were eluted from the

column by a linear gradient (200 ml) of 0–1 M NaCl in 0.05 M Tris/HCl (pH 8.0) at an NaCl concentration of approximately 0.2 M. Ammonium sulphate was added to the combined fractions containing *S*,*S*-IDS-degrading activity to a final concentration of 1 M. Precipitated proteins were removed by centrifugation ($5000 \times g$, 15 min). The supernatant was applied on a Phenyl-Superose HR column (1.0 by 10 cm; Pharmacia) pre-equilibrated with 1 M ammonium sulphate in 0.025 M Tris/HCl (pH 8.5) at a flow rate of 0.5 ml min^{-1}. The enzyme was eluted by a linear gradient (80 ml) of 1 to 0 M ammonium sulphate in 0.025 M Tris/HCl (pH 8.5) at a concentration of 0.4 M ammonium sulphate. Fractions containing *S*,*S*-IDS-degrading activity were pooled and then concentrated with Vivaspin concentrators (molecular weight cut off 10 kDa, Sartorius, Göttingen, Germany). The concentrated sample (0.5 ml) was applied on a gel filtration column (Superose 6 HR. 1.0 by 30 cm; Pharmacia) pre-equilibrated with 0.05 M Tris/HCl (pH 8.0) containing 0.1 M NaCl at a flow rate of 0.4 ml min^{-1}. All purification procedures were carried out at 6 °C. The purification procedure was monitored by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein concentrations were determined as described by Bradford (1976) with bovine serum albumine (Fluka) as the standard. The molecular mass of the subunit and the composition of the enzyme was determined using a 10% discontinuous SDS-polyacrylamide gel (Lämmli 1970) stained with Coomassie brilliant blue G-250 or silver (Shevchenko et al. 1996). The proteins of the low-molecular-weight marker kit (Pharmacia) were used as standards. The native weight of the enzyme was determined by analytical gel filtration as described above. The calibration curve was prepared with the Gel Filtration Calibration Kit (Pharmacia) for high- and low-molecular weight protein with the following protein standards: ferritin (450 kDa), catalase (232 kDa), aldolase (158 kDa) and bovine serum albumine (68 kDa).

Enzyme assays

The IDS-degrading activity was measured by adding 5 μl of enzyme to 1.6 ml containing 5 mM *S*,*S*-IDS in 0.05 M Tris/HCl (pH 8.0). Aliquots of 100 μl (for ion pair chromatography) or 380 μl (for photometric assay) were withdrawn as a function of time. In order to stop the reaction the samples were diluted with acidic copper sulphate solutions as described in the analysis of IDS. Enzyme assays were performed at 23 °C. For amino acid analysis the enzyme reaction was stopped by heating (3 min, 100 °C).

Analysis of IDS

For photometric analysis of IDS in enzyme assays, sample aliquots were 1:1 (v/v) diluted with 64 mM $CuSO_4$ (Reinecke et al. 2000). In contrast to the method of Reinecke et al., the pH of the copper solution was adjusted to 1.3 with phosphoric acid to avoid $Cu(OH)_2$ precipitation. Precipitated proteins were removed by centrifugation at $16{,}000 \times g$ for 5 min, and the absorption of the supernatant was measured at 710 nm. IDS showed a detection limit of 0.075 mM.

Ion pair chromatography of IDS and fumarate was performed on a Purospher® RP18 endcapped column (250×4 mm, Merck, Darmstadt, Germany) by using an isocratic eluent of 12.5% (v/v) methanol in formate buffer (15 mM sodium formate, 5 mM formic acid, and 2 mM tetrabutylammonium hydrogen sulphate). IDS was measured as Cu^{2+}-complex by diluting the samples 10:1 (v/v) with 200 mM copper sulphate (pH adjusted to 1.3 with phosphoric acid). Precipitated proteins were removed by centrifugation at $16{,}000 \times g$ for 5 min, and the supernatant was analysed. The detection wavelength of Cu^{2+}-IDS was 240 nm and the flow rate was 0.8 ml min^{-1}. Under these conditions *R*,*S*-IDS ($t_R = 8.2$ min, detection limit 0.05 mM) could be separated from *R*,*R*- and *S*,*S*-IDS ($t_R = 10.5$ min, detection limit 0.4 mM). The metabolite fumaric acid ($t_R = 12.5$ min) was identified by co-chromatography at 215 nm.

HPLC separation and analysis of D- and L-amino acids

D- and L-amino acids were separated and identified by precolumn derivatization with the FLEC/ADAM system ((+)-1-(9-fluorenyl)ethyl chloroformate/1-aminoadamantane) according to Einarsson & Josefsson (1987) on a Grom-Sil FLEC 1 250×4.0 mm column (Grom, Herrenberg, Germany). The amino acids were detected with a Waters 470 fluorescence detector (Millipore, Milford,

Mass., USA). The excitation wavelength was set at 263 nm and the emission analysed at 313 nm.

Analysis of ammonia

Ammonia concentrations were determined spectrophotometrically by use of a rapid test system (Microquant® 14750, Merck, Darmstadt, Germany).

Conversion by whole cells

As described above, cells of *R.* sp. SLRS7 were grown in mineral salts medium with the IDS isomer mixture (5 mM). IDS-grown cells were obtained by harvesting during exponential growth. The cells were resuspended in Na^+/K^+-phosphate buffer (50 mM; pH 7.4) to an optical density of 5.1 at 546 nm and incubated at 23 °C on a rotary shaker (125 rpm) with 6 mM *R,S*-IDS and 5.7 mM *S,S*-IDS. The consumption of the IDS isomers was followed by HPLC analysis.

Determination of kinetic parameters

The kinetic parameters were determined by using the Michaelis–Menten equation. The activity was measured at 23 °C as described above by adding 5 μl of enzyme from the Phenyl-Superose purification step to 1 ml containing concentration from 0.25 to 12.4 mM IDS in 0.05 M Tris/HCl (pH 8.0).

Substrate specificity

The activities towards different metal-IDS complexes and substrates (EDTA, EDDS, N,N′-(1,3-dicarboxypropyl)-L-arginin) were measured by ion pair chromatography as described in Enzyme assays using 5 mM substrate. All metal-IDS complexes were converted into the Cu^{2+}-complexes by treating the samples with the acidic copper sulphate solution. In case of Fe-IDS complexes, the samples were diluted 10:4 (v/v) with the copper sulphate solution.

Results

Biodegradability of IDS

An OECD 301F manometric respirometry test was performed to estimate the biodegradability of IDS. The test was carried out with an unadapted activated sludge inoculum from the municipal sewage treatment plant in Stuttgart-Büsnau. As shown in Figure 2 both test substances, the IDS-isomer mixture and the *R,S*-IDS are readily biodegraded. Remarkably, biodegradation of the isomer mixture did not reach the rate of the *R,S*-isomer. Therefore, we assumed that not all of the possible IDS-isomers are equally biodegraded. Consequently a biodegradation test with each of the three different isomers was set up. As shown in Figure 3, the *R,R*-isomer degradation rate was very low.

Enrichment and isolation of S,S-IDS and R,S-IDS degrading bacteria

Enrichment of IDS-degrading bacteria was performed in batch culture at 23 °C using mineral salts medium supplemented with 5 mM IDS isomer mixture as the sole source of carbon, nitrogen, and energy. Batch cultures were inoculated with sewage sludge from the municipal sewage treatment plant (Stuttgart-Büsnau, Germany). Enrichment cultures were plated and subcultivated on agar plates containing IDS isomer mixture. Colonies of different morphology were selected. Thus, seven different strains growing on IDS isomer mixture were isolated as pure cultures. Strain SLRS7 was selected for further investigations due to the fact that it exhibited very good growth on solid medium and could be handled in the lab without further safety restrictions. The BIOLOG

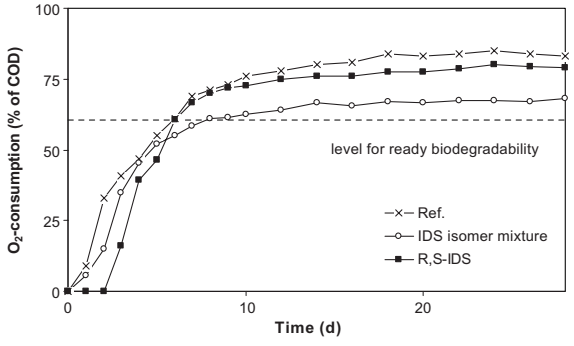

Figure 2. OECD 301F Manometric Respirometry Test. Biodegradation of the IDS-mixture and *R,S*-IDS by using activated sludge from the municipal sewage treatment plant (University Stuttgart, Büsnau, Germany) as inoculum. Sodium benzoate was used as reference (Ref.) compound. Data are expressed as % of the COD and represent the mean of duplicate values.

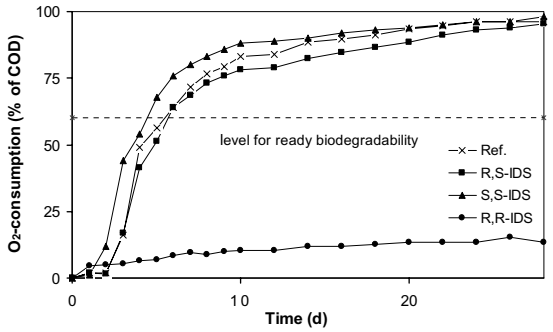

Figure 3. OECD 301F Manometric Respirometry Test. Biodegradation of the three IDS-stereoisomers by using activated sludge from the municipal sewage treatment plant (University Stuttgart, Büsnau, Germany) as inoculum. Sodium benzoate was used as reference (Ref.) compound. Data are expressed as % of the COD and represent the mean of duplicate values.

test system (GN microtitre plates for gram negative cells; BIOLOG Inc., Calif.) identified the strain SLRS7 with high probability as *Comamonas acidovorans* (similarity 0.624).

The strain SLRS7 was finally identified by sequencing the 16S-rDNA gene isolate. A sequence alignment with BLAST databases (Altschul et al. 1997) resulted in highest homologies with *Ralstonia taiwanensis* LMG 19424 & 19425 (accession number AF300324, AF300325) and the formerly assigned *Alcaligenes eutrophus* strain (M32021) with similarity of 98%. In the following the strain is referred to as *R*. sp. SLRS7. Further characterisation revealed the cells as motile rods of 1 μm size, gram-negative, and oxidase-positive.

IDS as a growth substrate

R. sp. SLRS7 was grown in mineral salts medium with *R*,*S*-IDS and *S*,*S*-IDS as the sole source of carbon, nitrogen, and energy. The strain grew on *S*,*S*-IDS or *R*,*S*-IDS as substrate at 23 °C with a generation time of 3.5 or 3.7 h, respectively. During growth on *S*,*S*-IDS and *R*,*S*-IDS, the pH increased as a result of ammonia excretion by the cells. The amount of ammonia released into the medium after 5 days corresponded to approximately 27% of the nitrogen originating from *S*,*S*-IDS and approximately 29% from *R*,*S*-IDS, respectively (Figure 4). Bacterial growth and a slight pH-increase was found when *R*,*R*-IDS was offered. But this was caused by *R*,*S*-IDS and fumaric acid which were both present as impurities in the *R*,*R*-IDS sample. For the growth curves, IDS-concentrations were measured by the rapid photometric test as described above. Growth at concentrations of more than 50 mM *R*,*S*-IDS and *S*,*S*-IDS was hindered, because the pH increased above 10.5. However growth was possible at concentrations even higher than 100 mM under pH-control within a range of 7.0 – 8.5 (data not shown).

Besides free IDS, it was tested if *R*. sp. SLRS7 could transform different metal-IDS complexes. Therefore, biodegradation tests in the presence of 5 mM Mg^{2+}-, Ca^{2+}-, Fe^{2+}-, Cu^{2+}-, or Mn^{2+}-*S*,*S*-IDS complexes were conducted. To ensure metal complexation of most of the *S*,*S*-IDS, the metal ions were added as sulphate salts in slight excess (5.2 mM) over *S*,*S*-IDS (5 mM). The

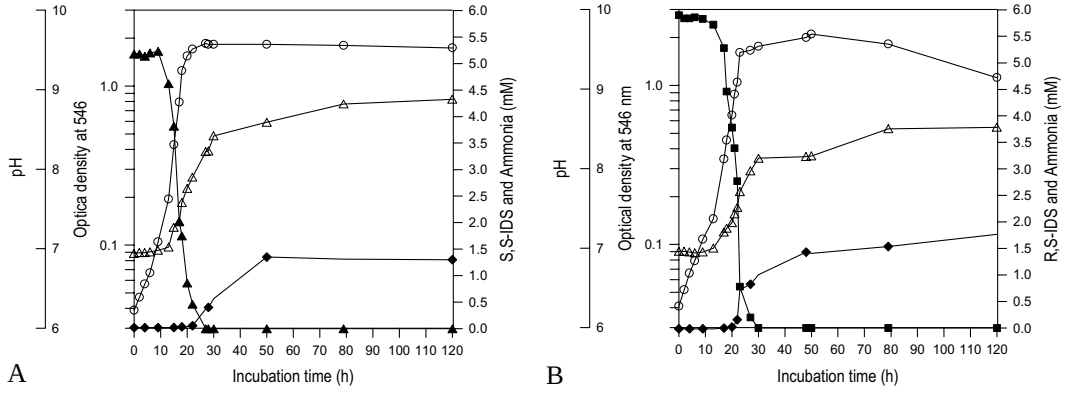

Figure 4. Growth of *R*. sp. SLRS7 in 500 ml Erlenmeyer flasks with 100 ml MSM containing *S*,*S*-IDS (A) or *R*,*S*-IDS (B) as the sole carbon, nitrogen, and energy source. The cultures were inoculated with a 10% (vol/vol) pre-culture grown with the respective substrate. Incubation temperature was 23 °C on a rotary shaker at 125 rpm. A: 5.2 mM starting concentration of *S*,*S*-IDS (▲); B: 5.9 mM *R*,*S*-IDS (■). Optical density (○), pH (△), and ammonia (◆).

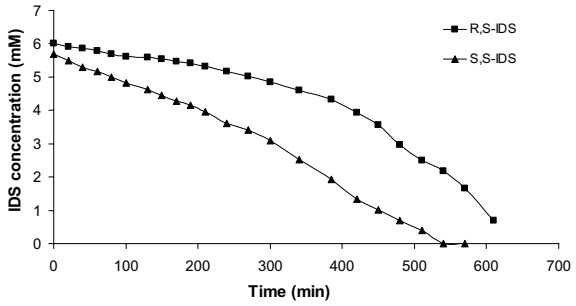

Figure 5. Simultaneous consumption of *R*,*S*-IDS and *S*,*S*-IDS by whole cells of *R*. sp. SLRS7. Cells were obtained by growth in mineral salts medium with IDS isomer mixture (5 mM). The cells were harvested, resuspended in phosphate buffer ($OD_{546} = 5.1$) and incubated at 23 °C with *R*,*S*-IDS plus 5.7 mM *S*,*S*-IDS. The concentration of IDS was determined by HPLC.

Mg^{2+}-, Ca^{2+}-, Mn^{2+}- and Fe^{2+}-IDS complexes were transformed largely within 3 days. In contrast only about 30% of the Cu^{2+}-*S*,*S*-IDS was degradable within 10 days (data not shown). Possibly Cu^{2+}-ions released during IDS-degradation inhibited microbial activity. Degradation was followed by monitoring the dissolved organic carbon because upon liberation of Mn^{2+}-, Fe^{2+}-, and Cu^{2+}-ions the respective hydroxides precipitated.

Experiments with whole cells of *R*. sp. SLRS7 incubated simultaneously with *R*,*S*-IDS and *S*,*S*-IDS showed that *S*,*S*-IDS concentration decreased approximately twice as fast as *R*,*S*-IDS (Figure 5). Interestingly, the *R*,*S*-IDS consumption increased rapidly after most of the *S*,*S*-IDS was consumed. From Figure 5 it cannot be deduced whether *S*,*S*- or *R*,*S*-IDS is the preferred substrate for uptake or initial transformation.

Enzyme purification

To identify the enzyme from *R*. sp. SLRS7 responsible for the initial transformation of IDS, the cell-extract was fractionated by using an anion exchange column, followed by hydrophobic interaction chromatography and gel filtration. The purification procedure is outlined in Table 1. The overall yield during purification was 7.2% and resulted in a 12.5-fold increase in specific activity. Cell-free extract of *R*. sp. SLRS7 grown with Nutrient-Broth (NB) medium had no IDS-degrading activity at all. This indicates enzyme induction by the substrate IDS.

Once purified, the enzyme was stable for at least 2 weeks at 6 °C without significant loss of activity. After 2 month storage at 6 °C however only 25% of the starting activity was measured. In this case, the starting activity could almost completely be restored by dithiotreitol treatment. To avoid loss of activity by freezing and thawing, the purified enzyme was divided in 50 μl portions, then treated with 1 mM dithiothreitol and frozen at −20 °C.

Molecular weight and subunit structure

The molecular mass of the native enzyme calculated by gel filtration was 240 kDa. The SDS-PAGE analysis gave a single protein band of approximately 57-kDa (Figure 6), indicating that the IDS-degrading enzyme is most likely a homotetramer.

Transformation of IDS

The pure enzyme degraded *S*,*S*-IDS with formation of fumaric acid and L-aspartic acid without requirement of any cofactors. In addition, it catalysed also the degradation of *R*,*S*-IDS, however

Table 1. Purification of an IDS transforming carbon–nitrogen lyase from the bacterial strain *Ralstonia* sp. SLRS7

Purification step	Vol (ml)	Total amt of protein (mg)	Sp act[a] (μmol of *S*,*S*-IDS/min/mg)	Yield (%)	Purification (fold)
Cell-free extract	56	1260	0.85	100	1
Ultracentrifugation	43	753	1.06	74.5	1.2
Q-Sepharose	23	250	2.74	64.0	3.2
Phenyl-superose	17	46.8	7.18	31.4	8.4
Superose 6	1.2	7.3	10.6	7.2	12.5

[a] The specific activity was determined by measuring the disappearance of *S*,*S*-IDS.

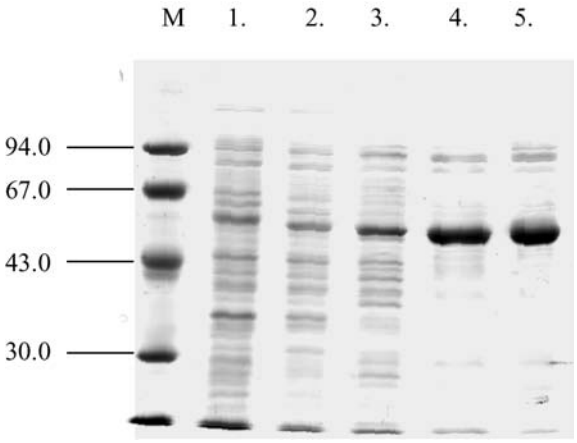

Figure 6. SDS-Page of the C—N lyase from *R*. sp. SLRS7 at different purification steps. Lanes: (1) crude extract; (2) crude extract after ultracentrifugation; (3) after Q-Sepharose; (4) active samples from Phenyl-Superose; (5) purified protein after gel filtration with Superose 6; M, molecular mass markers. Numbers on the left are molecular masses in kilodaltons. Lanes 1–5 were loaded with 5 μg protein.

only D-aspartic acid was liberated besides fumaric acid (Figure 7). The *R*,*R*-IDS isomer was not transformed by the enzyme. These results demonstrate that the IDS-degrading enzyme is a carbon–nitrogen lyase that catalyses a cleavage reaction of the C—N bond between the succinic and the aspartic acid residue. Interestingly, this non-hydrolytic cleavage of *R*,*S*-IDS led only to the formation of D-aspartic acid, indicating that the *S*-configuration of IDS is mandatory for the cleavage reaction.

The carbon–nitrogen lyase catalyses also the reverse reaction. An equilibrium constant K_{eq} of 81×10^{-3} for *R*,*S*-IDS and 120×10^{-3} for *S*,*S*-IDS was calculated according to the following equation $K_{eq} = c(\text{fumaric acid})^2/c(\text{IDS})$ (data not shown). Thus, the equilibrium for both reactions is clearly in favour of the side of the cleavage products fumaric acid and aspartic acid.

Enzymatic characterisation of the C—N lyase

The enzyme was active in a broad range from pH 6 to 11.5 with an optimum at pH 8–8.5. The maximum activity was found at 35 °C whereas no activity was observed at 58 °C. The determined kinetic parameters are shown in Table 2. The catalytic constant (k_{cat}) for *S*,*S*-IDS was approximately twice as high as for *R*,*S*-IDS and the catalytic efficiency (k_{cat}/K_m) of the two isomers exhibited approximately the same value. The pure enzyme showed a typical protein absorption spectrum with a maximum at 280 nm.

Metal-IDS chelates as substrates for the C—N lyase

It can be expected that after intended use IDS occurs most likely as metal-complex in waste water treatment facilities or the environment. Hence, it was tested whether metal-*S*,*S*-IDS chelates were transformed by the lyase. The metal-complexes were provided by combining equimolar concentrations of metal ions and *S*,*S*-IDS. To ensure

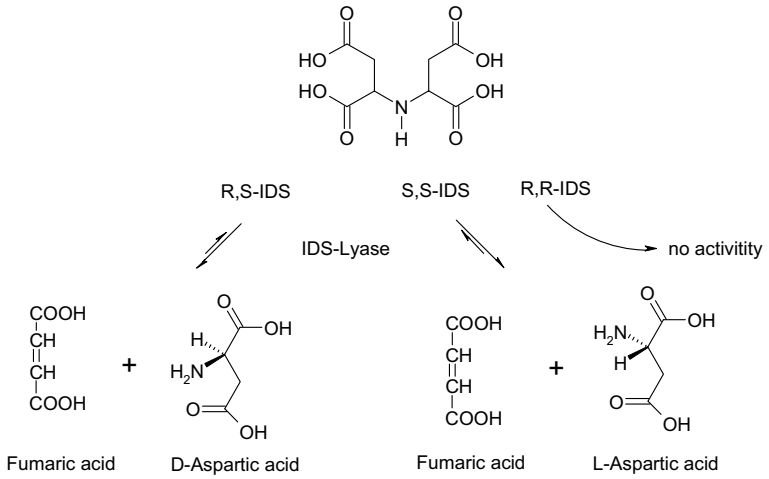

Figure 7. Initial transformation of IDS by *Ralstonia* sp. SLRS7.

Table 2. Kinetic parameters of the C—N lyase

Substrate	K_m (mM)	V_{max} (μmol/min)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})
S,*S*-IDS	2.5 (0.5)	270 (20)	79	3.2×10^4
R,*S*-IDS	1.0 (0.25)	170 (9)	49	4.9×10^4

The values in parentheses are standard deviations.

complete complexation with IDS, metal-sulphate (5.2 mM) was added in slight excess to *S*,*S*-IDS (5 mM). Among the different metal-complexes tested, Ca^{2+}-IDS was consumed with the highest specific activity. Mg^{2+}-IDS was also transformed but at lower rates. All other tested metal-IDS complexes (Mn^{2+}-, Zn^{2+}-, Cu^{2+}-, Fe^{2+}-, and Fe^{3+}-*S*,*S*-IDS) were transformed with a negligible rate or not at all (Table 3). This could be due to toxic effects of the metal ions or to the lack of activity towards these heavy-metal IDS complexes.

Further substrates for the C—N lyase

Activities with other chelating agents such as EDTA, EDDS isomer mixture and *S*,*S*-EDDS were tested. The activity with *S*,*S*-EDDS was measured, but showed only 1.4% of the *S*,*S*-IDS degrading activity (Table 3). In contrast N,N′-(1,3-dicarboxypropyl)-L-arginin (Nopaline), which harbours structural similarity to IDS and might be a potential natural substrate, was not transformed.

Liberation of ammonia

Ammonia, which was responsible for the pH increase during growth, must be a product from aspartate catabolism. The purified carbon–nitrogen lyase did not cleave L-/D-aspartic acid to form ammonia and fumaric acid, so that a second carbon–nitrogen lyase, probably an L-aspartate ammonia-lyase, must be induced during growth as it is well known for the aspartate metabolism.

Discussion

Initial biodegradation tests according to OECD guideline 301F with activated sludge inoculum from the municipal treatment plant in Stuttgart-Büsnau revealed 69% biodegradation after 28 days

Table 3. Acitvity of the C—N lyase from *Ralstonia* sp. SLRS7 with a variety of substrates

Substrate	Activity[a] (μmol min^{-1} mg^{-1})	Activity (% of activity with *S*,*S*-IDS)
S,*S*-IDS	7.18	100
Ca^{2+}-*S*,*S*-IDS	4.56	63.5
Mg^{2+}-*S*,*S*-IDS	2.66	37
Mn^{2+}-*S*,*S*-IDS	0.07	1
Fe^{3+}-*S*,*S*-IDS	0	No transformation
Fe^{2+}-*S*,*S*-IDS	0	No transformation
Zn^{2+}-*S*,*S*-IDS	0	No transformation
Cu^{2+}-*S*,*S*-IDS	0	No transformation
S,*S*-EDDS	0.1	1.4
Nopaline	0	No transformation
EDTA	0	No transformation

[a] Assays were performed as described in Materials and Methods.

indicating ready biodegradability of the IDS isomer mixture. An IDS-degrading strain was isolated from this sludge and identified. Reinecke et al. (2000) proposed two cleavage mechanisms for IDS degradation. One involves a monooxygenase system as shown for EDTA (Witschel et al. 1997) and NTA (Uetz et al. 1991). The second proposed a cleavage mechanism similar to that described by Witschel & Egli (1998) for EDDS. In the present work the IDS-degrading strain *R.* sp. SLRS7 confirmed an initial lyase-dependent degradation of IDS. The purified enzyme transformed *S*,*S*-IDS and *R*,*S*-IDS and in both cases led to the formation of fumaric acid. For *S*,*S*-IDS transformation the expected metabolite L-aspartic acid was detected, whereas *R*,*S*-IDS transformation led to D-aspartic acid. The metabolites were identified based on comparison with authentic standards. These results demonstrated that the enzyme cleaves only an *S*-configuration at the asymmetric carbon atom. Necessity of *S*-configuration was also observed for EDDS-degradation by strain DSM 9103 (Witschel & Egli 1998).

The IDS-degrading enzyme could be classified as a carbon–nitrogen lyase (EC 4.3.3.). Further metabolism of L-aspartic acid is supposedly based on an L-aspartate ammonia-lyase which generates fumaric acid and ammonia. Thus, the deamination of L-aspartic acid is responsible for the pH increase during growth on *S*,*S*-IDS. D-Aspartic acid metabolism probably starts with an aspartate

racemase which funnels D-aspartic acid into the L-aspartic acid metabolism. In whole cell conversion assays *S*,*S*-IDS consumption was approximately twice as fast as *R*,*S*-IDS. Supposedly this is due to the fact that the lyase attacks *S*,*S*-IDS on both *S*-configuration carbons, whereas *R*,*S*-IDS could only be attacked from one side. Therefore, the probability for cleavage is twice as high for *S*,*S*-IDS compared to *R*,*S*-IDS. Thus, *S*,*S*-IDS exhibited an approximately two times higher k_{cat} in comparison to *R*,*S*-IDS. The purified C—N lyase accepted free *S*,*S*-IDS as well as Ca^{2+}-, Mg^{2+}-, and to a negligible extend Mn^{2+}-*S*,*S*-IDS complexes as substrates but no Fe^{2+}, Fe^{3+}-, and Cu^{2+}-*S*,*S*-IDS. This indicates that heavy-metal complexes are not transformed by the purified enzyme. Whether this is due to the size of the complexes or to metal toxicity remains to be unravelled.

In contrast, strain SLRS7 degraded well Ca^{2+}-, Mg^{2+}-, Mn^{2+}-, and also Fe^{2+}-IDS, whereas only degradation of Cu^{2+}-IDS was poor. It must be pointed out that Cu^{2+}-IDS exhibited a complex-formation constant ($pK_{Cu\text{-}IDS}$) of 14.3, whereas Fe^{2+}-, Mn^{2+}-, Ca^{2+}-, and Mg^{2+}-IDS exhibited p*K*-values of only 8.2, 7.3, 6.7, and 6.0 (Bayer 1998). This strongly suggests that biodegradability of metal-IDS complexes by *R.* sp. SLRS7 depends on the concentration of free IDS. Consequently metal complexes with lower $pK_{Me\text{-}IDS}$-values are more easily biodegraded. Such relationship between the complex-formation constant and the biodegradability of the different metal complexes was also observed for EDDS by Witschel & Egli (1998). An explanation for this dependency could be that only free IDS can enter the cell. This is indicated by heavy-metal hydroxide precipitation during the biodegradation test with heavy-metal IDS complexes. If complexed IDS is the form of cell uptake, the question arises how bacteria can handle the amounts of metal ions entering the cell. An excretion of the metal ions could be considered. For EDTA transport into cells of strain DSM 9103 Witschel et al. (1999) reported that only free EDTA and complexes with low complex-formation constant were taken up. IDS uptake by strain SLRS7 should probably take place in a similar manner.

The activity of the C—N lyase was also tested with other chelating agents such as *S*,*S*-EDDS and EDTA. Only *S*,*S*-EDDS was transformed, but the measured activity differed from the EDDS-degrading lyase from strain DSM 9103 (Witschel et al. 1998) by three orders of magnitude. Consistently, both lyases only accept the *S*-configuration, they resemble in molecular mass and activity towards metal-complexes. A significant difference is the subunit organisation. The EDDS degrading-lyase from strain DSM 9103 is a homodimer in contrast to a homotetramer of the IDS-degrading system. Overall one can assume, that the two lyases belong to the same enzyme family (EC 4.3.3). The natural function of the C—N lyase remains unknown.

It will be of interest whether syntheses of new chemicals must be adapted to naturally occurring stereoselective cleaving enzymes in order to ensure biodegradability or if natural microbial communities provide alternative routes to circumvent stereoselective biodegradation. This is subject of further investigations.

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