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Production of D-proline from L-arginine using *Pseudomonas aeruginosa* [1]

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Abstract

Several microorganisms that can use (*S*)-5-[(amino-iminomethyl) amino]-2-chloropentanoic acid (L-Cl-arginine) as a nitrogen source have been isolated, the most interesting of which is a spontaneous mutant of *Pseudomonas aeruginosa* PAO1 (DSM 10581). In a fermenter, this unique biocatalyst hydrolysed L-Cl-arginine to (*S*)-5-amino-2-chloropentanoic acid (L-Cl-ornithine), which spontaneously converted to D-proline with inversion of configuration at an apparent average rate of 0.12 mmol $^{-1}$ h^{-1} OD^{-1}. The enzyme, for which we suggest the name Cl-arginine amidinohydrolase, was best induced by using the substrate L-Cl-arginine as inducer and L-arginine as nitrogen source. The results presented here describe a new route for the production of D-proline from L-arginine, involving a chemical step and a biocatalytic step followed by a spontaneous chemical cyclisation.

Keywords: D-Proline; *Pseudomonas aeruginosa*; Amidinohydrolase; (*S*)-5-[(Amino-iminomethyl) amino]-2-chloropentanoic acid; (*S*)-5-Amino-2-chloropentanoic acid

1. Introduction

Optically active amino acids are interesting intermediates for the synthesis of pharmaceuticals, cosmetics and pesticides, and the production of D-amino acids is currently of great interest [1]. D-Proline is an important intermediate for the production of several serotonin analogues [2], but although L-proline can be easily obtained by isolation from protein hydrolysates, D-proline is difficult to obtain from natural sources.

The normal biosynthetic routes to optically active amino acids involve acylases, hydantoinases, esterases or lipases, and have been discussed in several excellent articles [1,3–6]. The enantioselective hydrolysis of *N*-acetyl-amino acids by acylase I [EC 3.5.1.14] is the most widely used method for the large scale separation of enantiomers of amino acids. This method was developed on an industrial scale in the 1950s and 1960s by Tanabe Seijaku Co. Ltd. and processes for the production of L-methionine, L-valine, L-phenylalanine and other amino acids from their racemic *N*-acetyl derivatives have been established [7]. However, al-

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[1] Dedicated to Professor Hideaki Yamada in honor of his 70th birthday.

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though acylase I is reported to have a broad substrate spectrum, it does not react with the cyclic substrate *N*-acetyl-L-proline. New methods were therefore needed for the production of D-proline and D-proline derivatives.

Both biotechnological and chemical routes [8] to these compounds have been investigated, and several research groups have isolated new enzymes with enantioselective *N*-acetyl-L-proline acylase activity [9–13]. Sauter et al. at Lonza AG [14] have developed a resolution involving esterases. They isolated several microorganisms that were capable of hydrolysing various proline esters and amides. One of these strains, *Aureobacterium* sp. LS10 (DSM 10203), hydrolyses proline isopropylester with high enantioselectivity ($E > 100$). The same research group also established a process for the production of *N*-benzyloxycarbonyl-D-proline (Cbz-D-proline) from Cbz-DL-proline, using an enantioselective hydrolytic enzyme from *Arthrobacter* sp. HSZ5 (DSM10329) [15]. D-Proline can also be produced from DL-proline, using *Candida* or *Trichospora*; these strains are reported to use L-proline selectively as a carbon source [16]. Yagasaki and Ozaki [1] have reported a two step process for the production of D-proline from L-proline using a recombinant racemase in combination with an L-proline degrading *Candida* sp. However, all of these methods involve enzymatic resolution, resulting in a theoretical maximum yield of 50%.

Other routes which allow a theoretical maximum yield of 100% have also been developed. Several chemical routes have been established, one of which involves the racemisation of L-proline followed by asymmetric transformation of D-proline through the formation of the salt with (2*S*,3*S*)-tartaric acid, in which yields of 85% with 100% optical purity have been achieved [17]. Biosynthetic routes have also been established, such as that in which *Proteus mitajiri* (ATCC 21136) is cultivated in the presence of L-ornithine [18].

In this paper, we report a new biosynthetic route to D-proline starting from L-arginine [19]. This pathway is unique, and describes a new enzyme activity that can produce L-Cl-ornithine from L-Cl-arginine. Once formed, L-Cl-ornithine is spontaneously converted to D-proline with inversion of configuration.

2. Experimental

2.1. Materials

Compounds synthesised in the course of this work were identified by HPLC analysis using a Hewlett Packard 1050 instrument, and by ^{1}H NMR using a Varian Unity-400 MHz instrument. HPLC columns were from Macherey-Nagel. 2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosylisothiocyanate (GITC) was from Fluka. Optical rotation of chiral compounds was determined using a polarimeter (Propol). Fermentations were carried out in a 3.5 l fermenter (Chemap CF2000/FZ2000). Biotransformations using resting cells were carried out in a 1 l fermenter (Applikon ADI 1030).

All chemicals were from commercial sources or from Lonza AG unless otherwise indicated, and were used without further purification.

2.2. Enrichment and isolation of microorganisms

The enrichment and isolation of microorganisms was carried out using standard procedures. Soil samples or samples from the sewage treatment plant of Lonza AG were used as inocula for the enrichments. The enrichment media contained glucose, glycerol or L-Cl-arginine as carbon source at a maximum concentration of 20 g/l. As nitrogen source either L-arginine, urea, $(NH_4)_2SO_4$ or L-Cl-arginine up to a concentration of 10 mM was used. The enrichment cultures were incubated at 37°C with constant shaking in mineral salts medium ($MgCl_2 \cdot 6H_2O$, 0.4 g/l; $CaCl_2 \cdot 2H_2O$, 0.014 g/l; $FeCl_3 \cdot 6H_2O$, 2.8 mg/l; Na_2SO_4, 0.1 g/l;

Na_2HPO_4, 2 g/l; KH_2PO_4, 1 g/l; NaCl, 2 g/l), vitamin solution (pyridoxal · HCl, 10 mg/l; riboflavin, 5 mg/l; nicotinamide, 5 mg/l; thiamine · HCl, 5 mg/l; biotin, 2 mg/l; pantothenic acid, 5 mg/l; *p*-aminobenzoic acid, 5 mg/l; folic acid, 2 mg/l; cyanocobalamin, 5 mg/l) 1 ml/l; trace element solution (KOH, 15.1 g/l; EDTA · Na_2 · $2H_2O$, 100.0 g/l; $ZnSO_4 \cdot 7H_2O$, 9.0 g/l; $MnCl_4 \cdot 4H_2O$, 4.0 g/l; H_3BO_3, 2.7 g/l; $CoCl_2 \cdot 6H_2O$, 1.8 g/l; $CuCl_2 \cdot 2H_2O$, 1.5 g/l; $NiCl_2 \cdot 6H_2O$, 0.18 g/l; $Na_2MoO_4 \cdot 2H_2O$, 0.27 g/l) 1 ml/l. The pH was adjusted to 7.0. Full medium contained tryptone 10 g/l, meat extract 5 g/l, NaCl 5 g/l and yeast extract 5 g/l. Solid medium contained 20 g/l agar in addition to the above.

2.3. Isolation of a spontaneous mutant

Pseudomonas aeruginosa PAO1 [20] was plated on solid mineral salts medium containing 20 mM glycerol as carbon source and 5 mM L-Cl-arginine as nitrogen source. Plates were incubated at 37°C for 2–3 weeks.

2.4. Analytical methods

All potentially interesting strains isolated from the enrichment cultures were identified by the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM) and by the use of API-tests (bio Merieux SY, France). Proline was determined by HPLC using a Nucleosil 120-3 C_{18} 3 μm column (Macherey-Nagel). Prior to analysis, proline was derivatised with 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl-isothiocyanate (GITC) as follows: 30 μl sample was mixed with 30 μl 1.2% (v/v) triethylamine in H_2O, 30 μl of GITC solution (12 mg/ml in acetonitrile) was added and the reaction allowed to proceed for 12 min at room temperature. Derivatised samples were then stored at 4°C. The derivative was detected at a wavelength of 250 nm. TLC analysis of L-Cl-arginine, L-Cl-ornithine and proline was carried out using silica plates (Merck silica gel 60, F_{254}), with *n*-butanol:water:acetic acid (3:1:1) as solvent. Detection of Cl-arginine, L-Cl-ornithine, L-arginine and proline on TLC was carried out with fluoresceinisothiocyanate-ninhydrin reagent [21].

2.5. Growth of microorganisms, enzyme induction and biotransformation with resting cells

Growth media and incubation conditions are described above. To determine the optimal conditions for the induction of the enzyme, various carbon (glucose, glutamic acid, glutamine, agmatine ((4-aminobutyl)guanine) and arginine) and nitrogen (arginine, NH_4^+, ornithine, glutamic acid, citrulline, and betaine) sources were tested in minimal medium, together with various concentrations of L-Cl-arginine as inducer (Table 1). In some cases, the medium was supplemented with 0.1–0.2 g/l yeast extract.

After reaching the stationary growth phase, microorganisms were harvested by centrifugation (20 000 × *g* for 30 min) and washed with 10 mM Hepes or phosphate buffer, pH 7.0. Biotransformations were carried out in 100 mM Hepes or phosphate buffer, pH 7.0 to pH 8.5, with up to 150 mM L-Cl-arginine. Samples were withdrawn periodically, the biocatalyst removed by centrifugation and the supernatant analysed for D-proline by HPLC.

Determination of pH and temperature optima for the reaction were carried out using resting cells.

2.6. Production of D-proline in a fermenter

A preculture of *P. aeruginosa* (DSM 10581) was grown as described and was used to inoculate a 3.5 l fermenter. The fermentation medium contained $MgCl_2 \cdot 6H_2O$, 0.8 g/l; $CaCl_2 \cdot 2H_2O$, 0.16 g/l; $FeSO_4 \cdot 7H_2O$, 2.8 mg/l; Na_2SO_4, 0.1 g/l; Na_2HPO_4, 2g/l; KH_2PO_4, 1 g/l; glucose 10 g/l; L-Cl-arginine 6 mM; vitamin solution 2 ml/l; trace element solution

Table 1
Optimisation of growth of *P. aeruginosa* (DSM 10581) and induction of the Cl-arginine amidinohydrolase in the presence of different C- and N-sources and inducer concentrations

C-source (mM)	N-source[b] (mM)	L-Cl-arginine (mM)	Yeast extract (g/l)	OD_{650} 24 h	Cellular activity (%)[a]
Glucose 20	Arginine 1	4	0.2	1.7	100
Glucose 20	NH_4^+ 1	2	0.2	1.2	25
Glucose 20	Ornithine 1	1	0.1	1	10
Glucose 20	Glutamic acid 10	5	–	1.2	70
Glucose 20	Citrulline 1	1	0.1	0.9	6
Glucose 10	Betaine 10	5	–	1.2	70
Glucose 10	Arginine 1	5	–	0.8	70
Glycerol 20	Arginine 2	2	0.1	1	30
Glycerol 20	Ornithine 1	1	0.1	0.9	20
Glycerol 20	Citrulline 1	1	0.1	0.7	30
Glutamic acid 10	–	5	–	0.6	10
Glutamic acid 20	–	5	–	0.8	30
Glutamine 10	–	5	–	0.3	10
Glutamine 20	–	5	–	0.5	10
Arginine 10	–	5	–	0.5	70
Agmatine 10	–	5	–	0.2	20

[a]Cellular activity was determined by analysis of D-proline concentration, and thus represents the combined rate of the biotransformation and the chemical reaction.
[b]Could also function as a possible inducer.

1 ml/l (see above) and PPG 2000 4 ml/l. The fermenter was inoculated to give an initial OD_{650} of 0.2. A glucose feed of 2 g/l/h was started when the OD_{650} reached 2.8, and an additional 5 mmol/l of L-Cl-arginine was added after 20 h of fermentation. When the OD_{650} reached 16, the cells were harvested by centrifugation ($20\,000 \times g$ for 30 min) and resuspended in 10 mM Hepes buffer, pH 8.5, to an end volume of 1l and an OD_{650} of 21. L-Cl-Arginine was added periodically to the biotransformation to a final concentration of 225 mmol/l. The biotransformation was carried out in a 1l (working volume) fermenter at 37°C, pH 8.5, and the reaction was stopped after 40 h by cell removal.

The greenish cell-free supernatant (1100 ml) was filtered using Celite 535. Remaining proteins were precipitated with perchloric acid (50 ml of 60% v/v), and the solution was neutralised with KOH, followed by centrifugation (10 000 rpm for 5 min). Charcoal was added to remove colour, followed by filtration with Celite 535. The solution was concentrated tenfold using a rotary evaporator, chilled on ice, filtered through a paper filter and analysed for D-proline.

2.7. Derivatisation of D-proline to Cbz-D-proline

The derivatisation was carried out as described in Ref. [22]. 125 ml of D-proline solution, obtained as described above and containing 4.95 g D-proline, was chilled to 4°C before adding 9.3 g benzyl chloroformate stepwise. The pH was maintained at between 11.5 and 12 with 4 N NaOH. After neutralisation with HCl, the solution was extracted with butylacetate and the organic phase was discarded. The solution was acidified to pH 2 with HCl and re-extracted three times with butylacetate. The organic phases were combined and concentrated. Cbz-D-Proline crystallised out of the solution at 4°C when hexane was added.

2.8. Production of D-proline by a cell-free extract

Cells of *P. aeruginosa* were grown as described above, harvested in the late stationary phase by centrifugation ($20\,000 \times g$ for 30 min) and resuspended to an OD_{650} of 30 in 100 mM phosphate buffer, pH 8.5, containing 2 μl Benzonase (Merck). Cells were disrupted by pass-

ing three times through a French pressure cell at 120 MPa. Intact cells and cell debris were removed by ultracentrifugation (100 000 × *g* for 1 h). The enzyme activity in the cell-free extract was determined by mixing 1 ml of extract with 1 ml of 50 mM L-Cl-arginine in 100 mM phosphate buffer, pH 8.5, and incubating at 37°C. Intact cells were used as a positive control. Aliquots (200 μl) were removed from the assay mixture periodically, the reaction stopped by the addition of 10 μl perchloric acid (60% v/v), and the samples analysed by HPLC after neutralisation with KOH and centrifugation (10 000 rpm for 5 min).

2.9. Synthesis of L-Cl-arginine

The synthesis was carried out as described in the literature [23] with some minor modifications. Arginine · HCl (100 g) was dissolved in 150 ml of concentrated HCl. The solution was heated to 65°C and 75 ml of a 65% (v/v) solution of HNO_3 was added dropwise over a period of 30 min, during which time vigorous gas production was observed. The reaction was completed after a further 30 min at 65°C. The solution was concentrated and resuspended in 200 ml of concentrated HCl twice, before being finally concentrated to dryness. The yellowish crystals were dissolved again in 750 ml concentrated HCl and heated to 60°C.

L-Cl-Arginine crystallised out overnight at 4°C. The crystals were filtered and dried under vacuum. The yield was 66.7% (Smp. 149°C, α_D^{25} ($c = 10\%$ in H_2O) = −7.87°). ^1H NMR in ppm (400 MHz, in D_2O): 4.55 (dd, 1H, H-2); 3.25 (t, 2H, H-5); 2.15–1.95 (br. m, 2H); 1.8–1.7 (br. m, 2H), content: 100.8%, by titration.

3. Results

3.1. Isolation and identification of microorganisms

Microorganisms growing on L-Cl-arginine as nitrogen source and expressing the L-Cl-arginine amidinohydrolase were easily isolated from soil and industrial sewage. The microorganisms were identified as *P. cepacia*, *Agrobacterium radiobacter*, *Klebsiella pneumoniae* (DSM 10593) and *Arthrobacter* sp. (DSM 10582). None of these isolates have been described before.

When plates inoculated with wild-type *P. aeruginosa* PAO1, which was unable to grow on mineral salts medium with L-Cl-arginine as sole nitrogen source, were incubated for two weeks or more, colonies appeared. These bacteria were identified as *P. aeruginosa* (DSM 10581) by standard microbiological methods and differed from the wild-type only in their ability to utilise L-Cl-arginine as sole nitrogen source. On solid medium, colonies of this new isolate were brownish as opposed to greenish, as seen with the parent strain.

3.2. Microbial growth and enzyme induction

Arthrobacter sp. (DSM 10582) grew in both liquid full medium and in mineral salts medium containing glycerol as carbon source, with 10 mmol/l L-arginine and 0.5 mmol/l L-Cl-arginine as nitrogen source and/or inducer, to an optical density of $OD_{650} = 5.6$ and 5.1, respectively. Mineral salts medium containing glycerol as carbon source and L-Cl-arginine (1–5 mM) as nitrogen source allowed only poor growth (up to $OD_{650} = 0.6$). The biotransformation activity, analysed in resting cell experiments at pH 8.5 and 37°C, was highest in cells grown on full medium with 10 mM L-arginine as supplement, and was calculated to be 0.05 mmol l^{-1} h^{-1} OD^{-1} with an ee value for D-proline of $> 98\%$ as determined by HPLC.

Klebsiella pneumoniae (DSM 10593) grew on mineral salts medium containing 25 mmol/l glycerol as carbon source and 1 mmol/l L-Cl-arginine as nitrogen source to an $OD_{650} = 1.0$ at 37°C. In a resting cell biotransformation (100 mM Hepes buffer, pH 8.5, 37°C) L-Cl-arginine was converted to D-proline at a rate of 0.08 mmol l^{-1} h^{-1} OD^{-1}, with an ee value of $> 98\%$ as determined by HPLC.

P. aeruginosa (DSM 10581) grew on several different media, and the induction experiments were carried out as described in Table 1. The best growth and induction medium contained 20 mmol/l glucose as carbon source, with 1 mmol/l L-arginine and 4 mmol/l L-Cl-arginine as nitrogen source and inducer respectively, and was supplemented with 0.2 g/l yeast extract. Using cells grown on this medium, a biotransformation rate of 0.25 mmol l^{-1} h^{-1} OD^{-1} was observed in resting cell experiments carried out in 100 mM Hepes buffer, pH 8.5, at 37°C. As for the other strains, the optical purity of D-proline was $>98\%$ as determined by HPLC.

None of the organisms were able to grow on medium containing 10 mmol/l L-Cl-arginine as sole carbon source. This was probably due to the toxicity of this compound.

3.3. Preliminary analysis of the biotransformation with P. aeruginosa (DSM 10581)

The biotransformation was analysed in resting cell experiments with an OD_{650} of 17. The hydrolytic activity of the cells was 40% higher at 37°C than at 30°C. It was not possible to analyse the pH optimum of the enzymatic conversion of L-Cl-arginine to L-Cl-ornithine, since no quantitative analytical method for L-Cl-ornithine was available, and this reaction could not be separated from the cyclisation of L-Cl-ornithine to D-proline. The latter is a spontaneous, pH dependent chemical reaction with maximal rates at above pH 9.5. The pH optimum of the overall reaction was 8.5. Preliminary analysis of the enzyme activity showed that the initial rate in a cell-free system was at least 3 times greater than that seen with intact cells: cell-free extract produced D-proline at a rate of 16.2 mmol l^{-1} h^{-1}, and an analogous reaction with an equivalent amount of whole cells showed a rate of 5.1 mmol l^{-1} h^{-1}. This strongly suggests that the transport of L-Cl-arginine into the cell may be a rate-limiting step in the biotransformation.

3.4. Production of D-proline in a fermenter

Growing in a fermenter on mineral salts medium containing glucose, *P. aeruginosa* (DSM 10581) had a doubling time of 1 h (Fig. 1a). Glucose was consumed rapidly, and was therefore fed continuously with a feed rate of 2 g l^{-1} h^{-1}, starting after 6 h of growth. After 11.5 h of fermentation, the cells were harvested, resuspended to an OD_{650} of 21 in 10 mM Hepes buffer, pH 8.5 and transferred to a 1 l fermenter, where the biotransformation took place at 37°C. L-Cl-Arginine was added step-wise as shown in Fig. 1b. The total amount L-Cl-arginine added to the biotransformation was 225 mmol/l. The average biotransformation rate was 0.12 mmol l^{-1} h^{-1} OD^{-1}. The concentration of D-proline

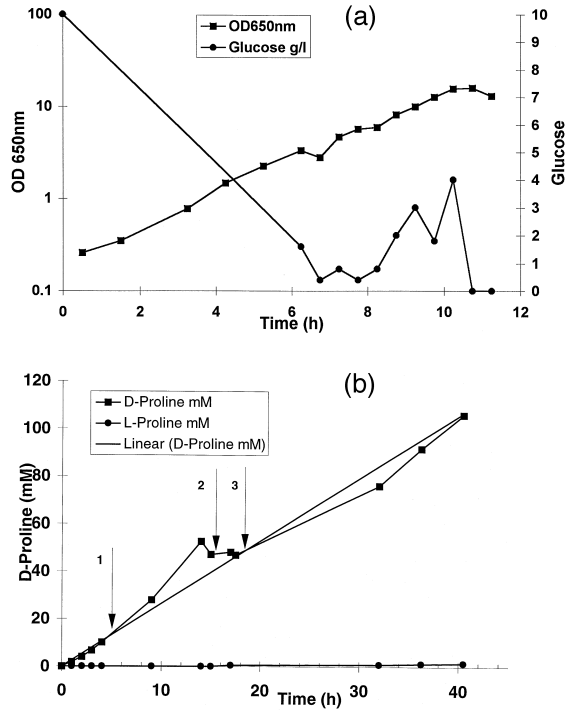

Fig. 1. (a) Growth and glucose consumption in *P. aeruginosa* (DSM 10581) during fermentation. (b) Biotransformation in a fermenter of L-Cl-arginine to D-proline with resting cells of *P. aeruginosa* (DSM 10581). Initial concentration of L-Cl-arginine in the fermenter was 50 mmol/l; the arrows indicate the addition of (1) 25 mmol/l (2) 50 mmol/l and (3) 100 mmol/l of L-Cl-arginine.

obtained at the end of the biotransformation was 106 mmol/l, with an ee of $>98\%$ as determined by HPLC. The biotransformation rate was linear over a time period of 40 h, after which time the reaction was stopped by cell removal since foaming was too strong to continue.

The cell-free solution was greenish, and protein rich due to cell lysis. After a rather complicated purification procedure, 125 ml of a 345 mmol/l D-proline solution (yield 19%, based on the amount of L-Cl-arginine in the biotransformation) with an ee value $>98\%$ was obtained.

3.5. Derivatisation of D-proline to Cbz-D-proline

In order to isolate the product, the clarified solution from the biotransformation containing 345 mmol/l D-proline was derivatised to give Cbz-D-proline as described above. After isolation and drying of the product, 3.6 g of Cbz-D-proline (yield 6.4%, based on the amount of L-Cl-arginine in the biotransformation) was obtained. The product was shown to be Cbz-D-proline by melting point, optical rotation, HPLC and NMR (melting point 68.5°C [22], content by titration with NaOH 97.8%, $[\alpha_D^{20}]$ ($c=2$ in acetic acid) $=+55.76°$, $[\alpha_{546}^{20}]$ ($c=2$ in acetic acid) $=+66.25$, ee by HPLC 95.4%. ^{1}H NMR (400 MHz in CD_3OD) δ in ppm: 7.35 (m, 5H); 5.1 (m, 2H); 4.3 (m,1H) 3.6–3.4 (m, 2H); 2.3–3.4 (m, 1H), 2.1–1.9 (m, 3H)).

4. Discussion

In this study microorganisms such as *Klebsiella*, *Agrobacterium*, *Arthrobacter* and *Pseudomonas* which were able to grow on L-Cl-arginine as sole nitrogen source were isolated. They expressed an enzyme which could convert L-Cl-arginine to L-Cl-ornithine. L-Cl-Arginine is known to inhibit growth of *P. aeruginosa* at a concentration of 2 mM when cells are grown on L-glutamate or L-ornithine [24] and our observation that L-Cl-arginine could only be used at low concentrations, i.e., as a nitrogen source but not as a carbon source, confirms this inhibitory effect. The best microorganism in terms of biotransformation rate and enantioselectivity, *P. aeruginosa* DSM 10581, was obtained through a spontaneous mutation of the wild-type *P. aeruginosa* PAO1. With this new biocatalyst, we were able to produce D-proline from L-arginine by the route shown in Fig. 2. In the first step, L-arginine was converted chemically to L-Cl-arginine, which was then hydrolysed by the biocatalyst to L-Cl-ornithine. In aqueous systems, L-Cl-ornithine undergoes a pH-dependent cyclisation reaction, yielding D-proline (data not shown). Thus L-Cl-ornithine was converted to D-proline in situ without the need for cell removal and isolation of the intermediate. D-proline was obtained with an enantiomeric excess of $>98\%$, and its identity was demonstrated by HPLC and by derivatisation to Cbz-D-proline. The overall isolated yield of this biotransformation as shown in Fig. 1b was very low, due to technical difficulties during the biotransformation and isolation. However, we are confident that these initial problems can be solved by conventional methods.

The biotransformation described in this study has not been reported before and we believe that it is catalysed by a novel enzyme, for which we suggest the name Cl-arginine amidinohydrolase. However, the possibility that the activity is the result of a spontaneous mutation in a known enzyme cannot be excluded. The utilisation of guanidino- and ureido- compounds as carbon and/or nitrogen sources by *Pseudomonas* has been well documented [25]. On the basis of its activity, the enzyme that we describe could be related to enzymes such as arginase [EC 3.5.3.1], guanidinoacetate amidinohydrolase [EC 3.5.3.2], guanidinobutyrate amidinohydrolase [EC 3.5.3.7], allantoate amidinohydrolase [EC 3.5.3.4] and agmatine amidinohydrolase [EC 3.5.3.11], or it could be derived from one of these enzymes. Obviously, the degradation of

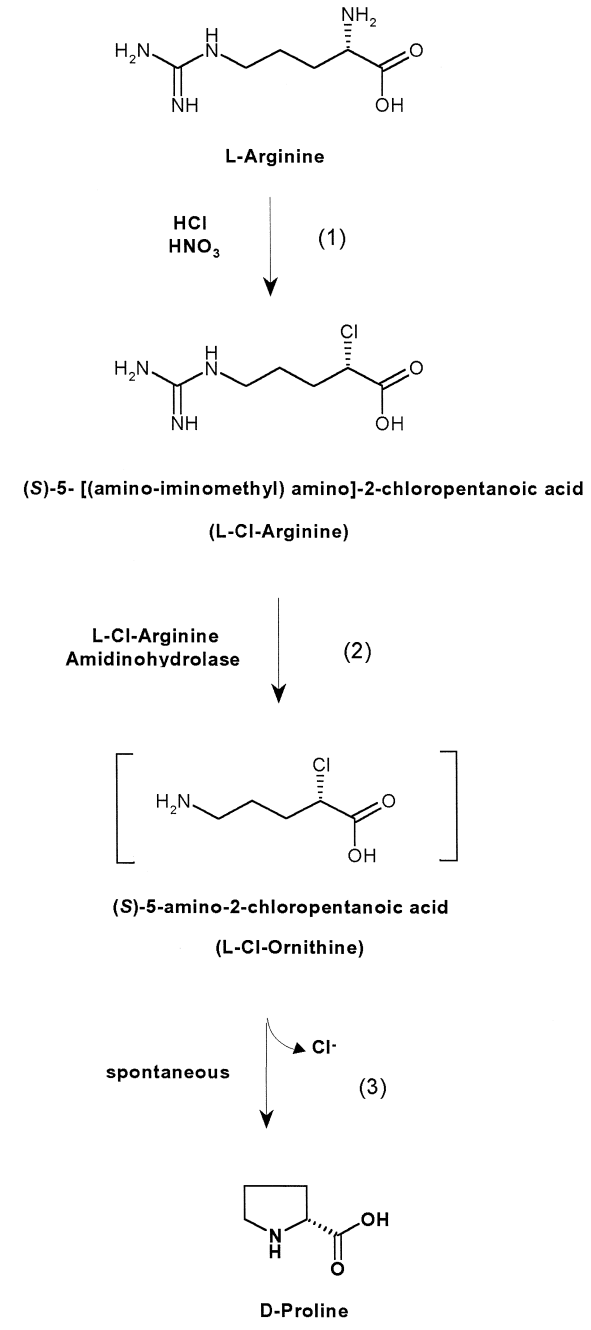

Fig. 2. Synthesis of D-proline from L-arginine with *P. aeruginosa* (DSM 10581), involving the chemical reaction of L-arginine to L-Cl-arginine (1) and a biotransformation catalysed by an L-Cl-arginine amidinohydrolase to an unstable intermediate, L-Cl-ornithine (2), followed by a spontaneous chemical cyclisation to D-proline (3).

L-Cl-arginine could be similar to the enzymatic hydrolysis of L-arginine catalysed by arginase. This reaction has been described for the production of L-ornithine from L-arginine with good yields [26]. Additionally, the biosynthesis of L-proline from L-ornithine seems initially very similar to the cyclisation of L-Cl-ornithine to D-proline. However, while the biosynthesis of L-proline is a three-step process [27], the transformation of L-Cl-ornithine to D-proline is a one-step, nonenzymatic, pH-dependent reaction. Several other degradation pathways for L-arginine are possible in *Pseudomonades* and these have been studied in detail [28,29]. Until the biotransformation that we describe has been analysed in more detail, the possibilities of a two step process, comparable to the arginine deiminase pathway or the arginine succinyltransferase pathway, still have to be considered. Recently, the enzyme that we describe has been partially purified in our laboratories (data not shown), and after two purification steps, the biotransformation activity was confined to one distinct fraction, strongly suggesting that only one enzyme is involved in this hydrolytic reaction.

The Cl-arginine amidinohydrolase is a soluble cytoplasmic enzyme, which is very active in cell-free extracts. Compared to a control experiment with resting cells, the initial biotransformation rate in the cell-free extract was more than three times higher, which implies that in whole cells a transport mechanism may be the rate limiting step in the biotransformation.

Interesting questions about the potential of this new synthetic pathway for the production of D-proline, the regulatory mechanisms of this new enzyme and about its evolutionary origin, are still open. We hope to address these aspects in the near future.

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