Research Article

Journal of Molecular Microbiology and Biotechnology

J Mol Microbiol Biotechnol 2005;9:16–25
DOI: 10.1159/000088142

Molecular Cloning and Biochemical Characterization of *L*-N-Carbamoylase from *Sinorhizobium meliloti* CECT4114

Sergio Martínez-Rodríguez Josefa María Clemente-Jiménez
Felipe Rodríguez-Vico Francisco Javier Las Heras-Vázquez

Departamento de Química-Física, Bioquímica y Química Inorgánica, I. Universidad de Almería, La Cañada de San Urbano, Almería, España

Key Words
N-Carbamoyl-*L*-amino acid amidohydrolase · *L*-N-carbamoylase · Hydantoinase process · Amino acid production

Abstract
An N-carbamoyl-*L*-amino acid amidohydrolase (*L*-N-carbamoylase) from *Sinorhizobium meliloti* CECT 4114 was cloned and expressed in *Escherichia coli*. The recombinant enzyme catalyzed the hydrolysis of N-carbamoyl α-amino acid to the corresponding free amino acid, and its purification has shown it to be strictly *L*-specific. The enzyme showed broad substrate specificity, and it is the first *L*-N-carbamoylase that hydrolyses N-carbamoyl-*L*-tryptophan as well as N-carbamoyl *L*-amino acids with aliphatic substituents. The apparent K_m values for N-carbamoyl-*L*-methionine and tryptophan were very similar (0.65 ± 0.09 and 0.69 ± 0.08 m*M*, respectively), although the rate constant was clearly higher for the *L*-methionine precursor (14.46 ± 0.30 s^{-1}) than the *L*-tryptophan one (0.15 ± 0.01 s^{-1}). The enzyme also hydrolyzed N-formyl-*L*-methionine ($k_{cat}/K_m = 7.10 \pm 2.52\ s^{-1} \cdot mM^{-1}$) and N-acetyl-*L*-methionine ($k_{cat}/K_m = 12.16 \pm 1.93\ s^{-1} \cdot mM^{-1}$), but the rate of hydrolysis was lower than for N-carbamoyl-*L*-methionine ($k_{cat}/K_m = 21.09 \pm 2.85$). This is the first *L*-N-carbamoylase involved in the 'hydantoinase process' that has hydrolyzed N-carbamoyl-*L*-cysteine, though less efficiently than N-carbamoyl-*L*-methionine. The enzyme did not hydrolyze ureidosuccinic acid or 3-ureidopropionic acid. The native form of the enzyme was a homodimer with a molecular mass of 90 kDa. The optimum conditions for the enzyme were 60°C and pH 8.0. Enzyme activity required the presence of divalent metal ions such as Ni^{2+}, Mn^{2+}, Co^{2+} and Fe^{2+}, and five amino acids putatively involved in the metal binding were found in the amino acid sequence.

Introduction

Optically pure *L*-amino acids are used as feed and food additives, as intermediates for pharmaceuticals, cosmetics, and pesticides, and as chiral synthons in organic synthesis [Bommarius et al., 1996]. Additionally, unnatural *L*-amino acids, which are not used in nature as building blocks for protein biosynthesis, are able to imitate the structure of natural amino acids in a type of molecular mimicry and thereby modulate the natural reaction, for example in the case of receptor interactions [Maier and Gaebert, 2003]. They are therefore used as a constituent

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F.J. Las Heras-Vázquez
Departamento de Química-Física, Bioquímica y Química Inorgánica
Edificio C.I.T.E I., Universidad de Almería, La Cañada de San Urbano
ES–04120 Almería (Spain)
Tel. +34 950 015055, Fax +34 950 015615, E-Mail fjheras@ual.es

of many drugs as protease inhibitors for preventing HIV infection, peptide ligands for the fibrinogen receptor, effective tachykinin antagonists, and neuronal receptor ligands [Li et al., 1999].

N-carbamoyl-*L*-amino acid amidohydrolase (*L*-N-carbamoylase) enzyme has been found in several microorganisms of the genera *Alcaligenes* [Ogawa et al., 1995], *Arthrobacter* [Gross et al., 1987], *Bacillus* [Yamashiro et al., 1988], *Flavobacterium* [Yokozeki et al., 1987] and *Pseudomonas* [Buchanan et al., 2001]. Although its biological function is still unknown [Pietzsch and Syldatk, 2002], from the biotechnological point of view *L*-N-carbamoylase plays an important role in the production of optically pure *L*-amino acids by an enzymatic method named 'hydantoinase process' [Altenbuchner et al., 2001]. This process is a cheap and environment-friendly enzymatic method for the potential production of any optically pure natural or unnatural *L*-amino acid from a wide spectrum of *D,L*-5-monosubstituted hydantoins used as substrate [Kim and Kim, 1995]. In this cascade of reactions, the chemically synthesized *D,L*-5-monosubstituted hydantoin ring is first hydrolyzed by a stereoselective *L*-hydantoinase enzyme. Further hydrolysis of the resulting enantiospecific N-carbamoyl α-*L*-amino acid to the corresponding free *L*-amino acid is catalyzed by highly enantiospecific N-carbamoyl α-amino acid amidohydrolase (N-carbamoylase). At the same time as hydantoinase hydrolyses the enantiospecific *L*-5-monosubstituted hydantoin in the first step, the chemical and/or enzymatic racemization of the remaining non-hydrolyzed *D*-5-monosubstituted hydantoin starts.

The *L*-N-carbamoylases have been classified into two groups depending on substrate specificity. One group mainly recognizes and hydrolyses N-carbamoyl-*L*-aromatic amino acids [Syldatk et al., 1987; Yokozeki et al., 1987], while the second one shows broad substrate specificity toward N-carbamoyl-*L*-aliphatic and aromatic amino acids [Ishikawa et al., 1993; Yamashiro et al., 1988]. However, *L*-N-carbamoylases that hydrolyze N-carbamoyl-*L*-tryptophan as well as N-carbamoyl *L*-amino acids with aliphatic substituents have not been reported [Hu et al., 2003]. This broad substrate specific *L*-N-carbamoylase could increase the commercial application of the 'hydantoinase process' currently used for obtaining *D*-amino acids, allowing *L*-amino acids to also be obtained [May et al., 2002]. The possibility of reducing production costs by using recombinant cells of *Escherichia coli*, thus increasing activity by overexpression of the enzymes involved in this reaction could constitute a commercially feasible process for *L*-amino acid production. To this end, we have characterized the hydantoin racemase gene of *Sinorhizobium meliloti* [Martinez-Rodriguez et al., 2004].

In the present work, we report the cloning and overexpression of an *L*-N-carbamoylase gene from *S. meliloti* CECT 4114 in *E. coli*. The purification and the enzymatic properties of the recombinant enzyme such as substrate specificity, kinetic parameters and reaction conditions are studied. These properties are compared with those of previously reported *L*-N-carbamoylase enzymes from different strains.

Results

Sequence Analysis of L-*N-Carbamoylase*

S. meliloti strains CECT 4114 and 4857 presented a specific fragment for the *L*-N-carbamoylase gene. Nucleotide sequence analysis showed 100% homology between these two fragments. The deduced amino acid sequence of the *S. meliloti* enzyme was compared with the sequences of *L*-N-carbamoylase having proven activity from different sources (fig. 1). The highest amino acid sequence identity was found between the studied *L*-N-carbamoylase and N-carbamoyl-*L*-cysteine amidohydrolase from *Pseudomonas* sp. strains BS (42.42%) [Shiba et al., 2002] and ON-4a (39.17%) [Ohmachi et al., 2002]. When compared with both *Bacillus stearothermophilus* strains (NCIB8224 and NS1122A) [Batisse et al., 1997; Mukohara et al., 1993] and *Bacillus kaustophilus* CCRC11223 [Hu et al., 2003], the percentage was slightly lower (36.74, 36.25 and 35.28%, respectively). The identity percentage when compared with *Arthrobacter aurescens* DM3747 *L*-N-carbamoylase [Wilms et al., 1999] was 32.05%. The lowest identity percentage was 29.19%, obtained when comparing the studied *S. meliloti* sequence with *L*-N-carbamoylase from *Pseudomonas* sp. NS671 [Watabe et al., 1992]. A high identity percentage was also found between the sequences of the studied enzyme and a β-alanine synthase enzyme (N-carbamoyl-β-alanine amidohydrolase) from *Saccharomyces kluyveri,* whose three-dimensional structure has recently been resolved [Lundgren et al., 2003] (35.47%).

Cloning, Functional Expression and Purification

The *S. meliloti L*-N-carbamoylase gene was amplified by PCR without the signal peptide-encoding sequence. Additionally, and in order to avoid a fusion protein between the *L*-N-carbamoylase gene and the N-terminal end of the β-galactosidase gene present in the pBSK vector, a TGA codon was included upstream from the ribosome

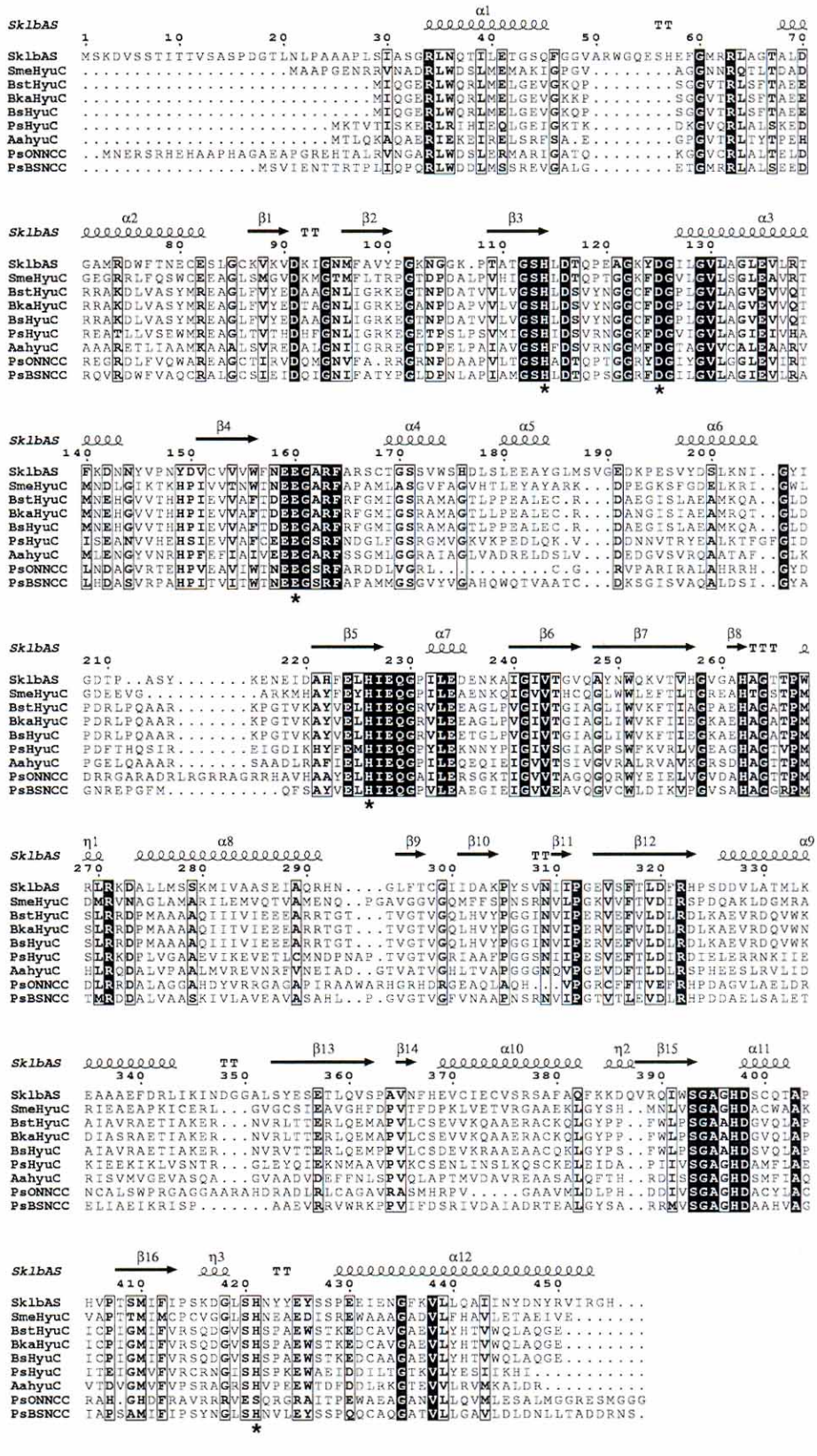

Fig. 1. Multiple alignments of the amino acid sequences of *L*-N-carbamoylases. β-Alanine synthase from *S. kluyveri* (SklbAS), GenBank accession No. AF333185; *L*-N-carbamoylase from *S. meliloti* (SmeHyuC), GenBank accession No. AY646850; *L*-N-carbamoylase from *B. stearothermophilus* NCIB8224 (BstHyuC), GenBank accession No. Y08752; *L*-N-carbamoylase from *B. kaustophilus* CCRC11223 (BkaHyuC), GenBank accession No. AF428538; *L*-N-carbamoylase from *B. stearothermophilus* NS1122A (BsHyuC), GenBank accession No. S67784; *L*-N-carbamoylase from *Pseudomonas* sp. NS761 (PsHyuC), GenBank accession No. M72717; *L*-N-carbamoylase from *A. aurescens* DSM 3747 (AaHyuC), GenBank accession No. AF146701; *N*-carbamoyl-*L*-cysteine amidohydrolase from *Pseudomonas* sp. ON-4a (PsONNCC), GenBank accession No. AB029899; N-carbamoyl-*L*-cysteine amidohydrolase from *Pseudomonas* sp. BS (PsBSNCC), GenBank accession No. AB070707. Alpha helices (α) and 3-helices (3_{10}, η) are displayed as medium and small squiggles, respectively. Beta strands (β) are rendered as arrows, strict beta turns as TT letters and strict alpha turns as TTT.

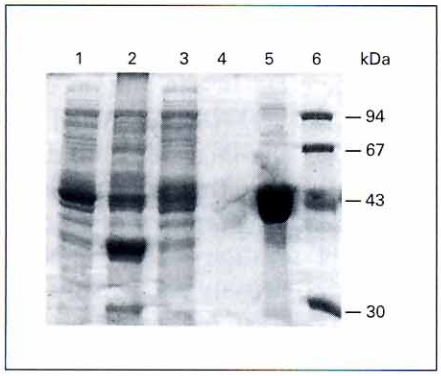

Fig. 2. SDS-PAGE analysis of each purification step of *S. meliloti* CECT 4114 *L*-N-carbamoylase from *E. coli* BL21 harboring the pSER35 plasmid. Lanes 1 and 2, supernatant and pellet of the resuspended crude extract after cell sonication; lane 3, eluate after adding the sonicated supernatant to the metal affinity column; lane 4, flow-through after washing the metal affinity column with buffer; lane 5, purified enzyme; lane 6, low molecular weight marker.

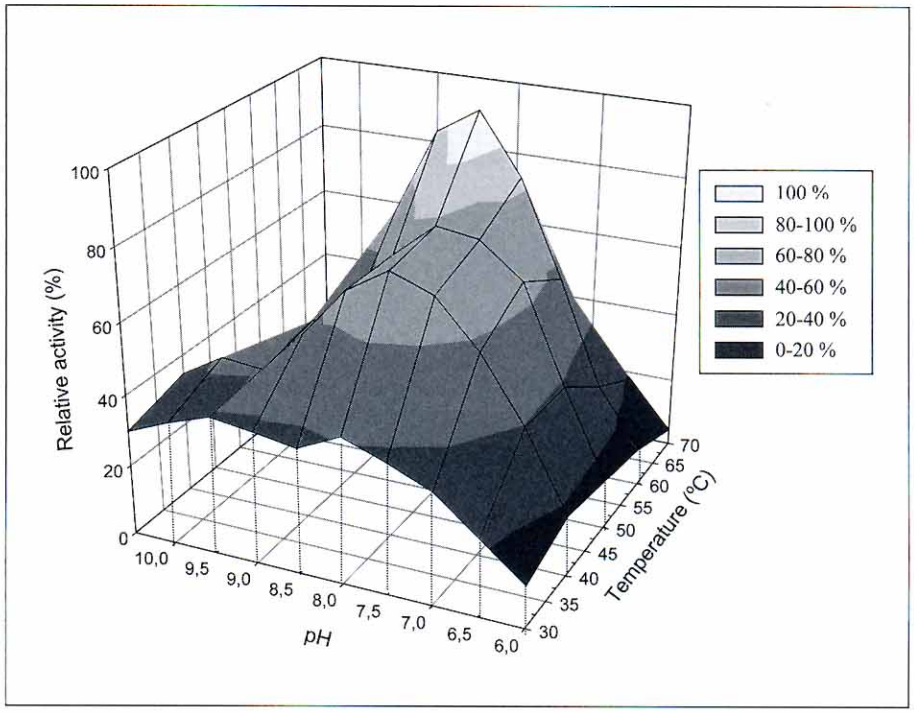

Fig. 3. Analysis of pH and temperature dependence in *S. meliloti* CECT 4114 *L*-N-carbamoylase activity. Reactions were carried out at pH 6.0–11.0 and temperatures from 30 to 70°C for 20 min with N-carbamoyl-*L*-methionine as substrate. The protein concentration used in the reaction was 5 μM. 100% of relative activity corresponded to 0.70 ± 0.05 U mg^{-1}.

binding site sequence and the beginning of the gene in SmLcar5 primer. The resulting plasmid containing the *L*-N-carbamoylase gene (1251 bp) was named pSER35. The *L*-N-carbamoylase gene was functionally expressed in *E. coli* BL21. Its activity was determined in crude extracts by high performance liquid chromatography (HPLC) using N-carbamoyl-*D* and *L*-methionine as substrate (see 'Experimental Procedures'), showing strict *L* enantioselectivity. A one-step purification procedure of the recombinant *L*-N-carbamoylase fused to the His_6 tag was employed using immobilized cobalt affinity chromatography. SDS-PAGE analysis indicated that the purified enzyme was over 95% pure after elution of the affinity column (fig. 2). Specific activity of the purified enzyme using N-carbamoyl-*L*-methionine as substrate was 0.66 U/mg. In 0.2 *M* sodium phosphate pH 7.5 the enzyme was stable at 4°C for 4 weeks, and in the same buffer with 20% glycerol the purified enzyme could be stored at –20°C over 3 months without noticeable loss of activity. The purified enzyme was active after 5 freeze-thaw cycles.

Molecular Mass and Subunit Structure

SDS-PAGE analysis of the purified *L*-N-carbamoylase enzyme gave an apparent molecular mass of approximately 40–42 kDa (fig. 2). The relative molecular mass of the native enzyme from pH 6.0–9.0 was estimated to be 90 kDa by size exclusion chromatography on a Superdex 200 HR column (data not shown). The molecular mass deduced from the amino acid sequence was 44,857 Da. These results suggest that the *S. meliloti* *L*-N-carbamoylase enzyme has a homodimeric structure.

Influence of pH and Temperature on Enzyme Activity

L-N-carbamoylase activity was determined in sodium phosphate, Tris/HCl and carbonate/bicarbonate buffers at a concentration of 0.2 *M* and pH values of 6.0–8.0, 8.0–9.0 and 9.0–10.5, respectively. In all cases enzyme activity was evaluated at temperatures ranging from 30 to 70°C. Maximum enzymatic activity proved to be at pH 8.0 and 60°C (fig. 3). However, thermal stability was found to be low when the enzyme was incubated at temperatures from 20 to 70°C (see 'Experimental Proce-

Table 1. Effect of metal ions and chemical agents on the activity of *S. meliloti L*-N-carbamoylase

Compound	Specific activity U mg^{-1}	Relative activity %
None	0.67 ± 0.03	100
Pb^{2+}	0.39 ± 0.03	58
Ca^{2+}	0.69 ± 0.04	103
Fe^{3+}	0.44 ± 0.02	66
Fe^{2+}	2.03 ± 0.01	303
Hg^{2+}	0	0
Mn^{2+}	3.24 ± 0.02	484
Zn^{2+}	0.29 ± 0.01	43
Co^{2+}	2.27 ± 0.07	339
Cu^{2+}	0.20 ± 0.01	30
Ni^{2+}	9.79 ± 0.12	1,461
Na^{+}	0.57 ± 0.03	85
K^{+}	0.56 ± 0.01	84
DTNB	0.39 ± 0.01	58
EDTA	0	0
DTT	0.67 ± 0.01	100
β-mercaptoethanol	0.44 ± 0.05	66
EDTA + Ni^{2+}	9.69 ± 0.09	1,446
EDTA + Mn^{2+}	3.12 ± 0.33	466
EDTA + Co^{2+}	2.33 ± 0.05	348
EDTA + Fe^{2+}	2.01 ± 0.29	300
EDTA + Ca^{2+}	0	0

The purified enzyme was incubated with the chloride salt of the metal ions, reducing, sulfhydryl or chelating reagents at room temperature in 0.2 *M* sodium phosphate buffer (pH 8.0) for 45 min. The restoration of *L*-N-carbamoylase activity by metal ions after treatment with EDTA chelating agent was also studied. The enzyme was incubated overnight with 10 m*M* of EDTA at 4°C. The chelating agent was removed by dialysis in four stages at 12-hour intervals, all at 4°C. Divalent metal salt solutions were added and incubated at room temperature for 45 min. Activity assays were carried out in triplicate as described in 'Experimental Procedures' with N-carbamoyl-*L*-methionine as substrate.

dures'), with gradually decreasing activity at temperatures over 30°C for 30 min, and only 20% activity remaining after incubation at 50°C for 30 min.

Effects of Metal Ions and Chemical Agents

Activity of the purified *L*-N-carbamoylase enzyme was assayed in the presence of 2 m*M* of different metal ions using N-carbamoyl-*L*-methionine as substrate (table 1). Incubation of the enzyme with some divalent metals such as Hg^{2+} caused total inhibition. Cu^{2+}, Zn^{2+}, Pb^{2+} and Fe^{3+} caused strong inhibition, while Na^{+} and K^{+} caused only slight inhibition. However, metal ions such as Ni^{2+}, Mn^{2+}, Co^{2+} and Fe^{2+} greatly enhanced activity. Enzyme activity was not affected by Ca^{2+} nor by the reducing compound dithiothreitol (DTT) at 10 m*M*. However, β-mercaptoethanol reducing compound caused 34% inhibition. 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) sulfhydryl reagent caused 42% inhibition. The enzyme showed no activity in the presence of 10 m*M* ethylenediaminetetraacetic acid (EDTA). However, after removal of the chelating agent by dialysis and the addition of 2 m*M* of Ni^{2+}, Mn^{2+}, Co^{2+} and Fe^{2+}, enzyme activity was greatly enhanced. These metal ions increased *L*-N-carbamoylase activity at concentrations from 0.5 m*M*, and no decrease in activity was detected at higher cation concentrations (5 m*M*).

Substrate Specificity and Kinetic Characterization

The ability of the purified *L*-N-carbamoylase to hydrolyze different substrates was examined. To this end, kinetic parameters K_m, k_{cat} and k_{cat}/K_m were obtained from hyperbolic saturation curves by least-squares fit of the data to the Michaelis-Menten equation (table 2). Reactions were carried out with different concentrations of optically pure and racemic substrates at 40°C after preincubation of the protein with 2 m*M* $NiCl_2$. *S. meliloti* CECT 4114 *L*-N-carbamoylase showed activity towards aliphatic and aromatic N-carbamoyl-*L*-specific derivatives, while the enzyme did not hydrolyze N-carbamoyl-*D*-amino acids. Apparent K_m values of N-carbamoyl-*L*-methionine and N-carbamoyl-*L*-tryptophan were almost identical (0.69 and 0.65 m*M*, respectively). However, k_{cat} value for the former was considerably higher (14.46 s^{-1}) than for the latter (0.15 s^{-1}). *L*-N-Carbamoylase showed activity for N-carbamoyl-*L*-cysteine with a higher k_{cat}/K_m value than for N-carbamoyl-*L*-tryptophan (2.76 as compared to 0.23 s^{-1} mM^{-1}). The enzyme was very active toward N-acetyl and N-formyl-*L*-amino acids with k_{cat}/K_m values of 12.16 and 7.10 s^{-1} mM^{-1}, respectively. Finally, 3-ureidopropionic acid (N-carbamoyl-β-alanine) and ureidosuccinic acid (N-carbamoyl-*D*,*L*-aspartic acid) were not recognized as substrates for *L*-N-carbamoylase.

Discussion

The search for new processes for the production of optically pure natural or nonnatural *L*-amino acids and the application of biological tools in the development of novel biocatalyst systems have required the use of recombinant *E. coli* cells overexpressing the enzymes involved in the 'hydantoinase process'. Additionally, *L*-N-carbamo-

Table 2. Substrate specificity and stereoselectivity of purified *S. meliloti* CECT 4114 *L*-N-carbamoylase

Substrate	K_m, m*M*	k_{cat}, s^{-1}	k_{cat}/K_m $s^{-1} \cdot mM^{-1}$
N-Carbamoyl-*L*-methionine	0.69 ± 0.08	14.46 ± 0.30	21.09 ± 2.85
N-Carbamoyl-*D*-methionine	not detected		
N-Acetyl-*L*-methionine	5.50 ± 0.63	66.84 ± 2.89	12.16 ± 1.93
N-Formyl-*L*-methionine	12.90 ± 3.14	91.66 ± 10.20	7.10 ± 2.52
N-Carbamoyl-*L*-cysteine	4.91 ± 0.72	13.55 ± 0.46	2.76 ± 0.49
N-Carbamoyl-*L*-glutamic acid	51.23 ± 5.83	1.52 ± 0.15	0.03 ± 0.00
N-Carbamoyl-*L*-valine	34.47 ± 3.48	16.90 ± 0.61	0.49 ± 0.07
N-Carbamoyl-*D*-valine	not detected		
N-Carbamoyl-*L*-phenylalanine	2.61 ± 0.49	4.26 ± 0.15	1.63 ± 0.37
N-Carbamoyl-*D*-phenylalanine	not detected		
N-Carbamoyl-*L*-tryptophan	0.65 ± 0.09	0.15 ± 0.01	0.23 ± 0.03
N-Carbamoyl-*L*-tyrosine	4.80 ± 0.47	0.30 ± 0.02	0.06 ± 0.00
N-Carbamoyl-*L*-alanine	0.94 ± 0.12	2.59 ± 0.15	2.76 ± 0.52
N-Carbamoyl-*L*-leucine	not detected		
N-Carbamoyl-*D*-leucine	not detected		
N-Carbamoyl-*L*-isoleucine	not detected		
N-Carbamoyl-*L*-serine	not detected		
N-Carbamoyl-*L*-*p*-hydroxy-phenylglycine	not detected		
N-Carbamoyl-*D*,*L*-α-aminobutyric acid	not detected		
N-Carbamoyl-*D*,*L*-aspartic acid	not detected		
3-Ureido propionic acid (*N*-carb-β-alanine)	not detected		

The kinetic parameters of *S. meliloti L*-N-carbamoylase were determined at 40°C for 20 min at pH 8.0 after preincubation with 2 m*M* Ni^{2+}. Reactions were performed in triplicate and the maximum substrate concentration was 100 m*M* for all substrates except for N-carbamoyl-*L*-glutamic acid and N-carbamoyl-*L*-valine, for which it was 160 m*M*.

ylases are so unstable that only three have been purified to homogeneity [Ogawa et al., 1995; Wilms et al., 1999]. The present study has determined the physical, biochemical and kinetic properties of *L*-N-carbamoylase from *S. meliloti* CECT 4114 after cloning, overexpression in *E. coli* and purification.

The amino acid sequence of *S. meliloti L*-N-carbamoylase showed higher homology compared with sequences from *Bacillus* genus strains than with sequences from *A. aurescens* DSM 3747 [Wilms et al., 1999] and *Pseudomonas* sp. NS 671 [Watabe et al., 1992]. However, it showed the highest homology when compared with both N-carbamoyl-*L*-cysteine amidohydrolases from *Pseudomonas* sp. strains ON-4a and BS [Ohmachi et al., 2002; Shiba et al., 2002]. The *L*-N-carbamoylase genes from S. *meliloti* and *Pseudomonas* sp. NS 671 together with both N-carbamoyl-*L*-cysteine amidohydrolases from *Pseudomonas* sp. strains ON-4a and BS had an ATG start codon [Ishikawa et al., 1993; Ohmachi et al., 2002; Shiba et al., 2002]. On the other hand, the three sequences from *Bacillus* genera strains and *A. aurescens* DSM 3747 started with the TTG and GTG codons, respectively [Batisse et al., 1997; Mukohara et al., 1993]. The apparent molecular mass of the subunit for all the recombinant *L*-N-carbamoylase enzymes is approximately 45 kDa, with the exception of *Alcaligenes xylosoxidans* (65 kDa) [Ogawa et al., 1995]. The relative molecular mass of *S. meliloti* CECT 4114 *L*-N-carbamoylase native enzyme was the same as that for the previously reported enzymes with a homodimeric structure [Ishikawa et al., 1993; Ogawa and Shimizu, 1994; Wilms et al., 1999]. In contrast, N-carbamoyl-*L*-cysteine amidohydrolase from *Pseudomonas* sp. ON-4a has recently been characterized as a homotetramer [Ohmachi et al., 2004].

The optimal physical parameters of *S. meliloti L*-N-carbamoylase (60°C and pH 8.0) were in the same range as those of the other enzymes. Previous works have found optimum reaction temperatures of 35–40°C for *Pseudomonas* sp. NS 671 and *A. xylosoxidans* [Ishikawa et al., 1993; Ogawa et al., 1995], and 50°C for *A. aurescens* DSM

3747 [Wilms et al., 1999] and N-carbamoyl-*L*-cysteine amidohydrolase from *Pseudomonas* sp. ON-4a [Ohmachi et al., 2004]. The optimum temperature rises to 70°C for the thermostable microorganisms of *B. stearothermophilus* NS 1122A [Ishikawa et al., 1994] and *B. kaustophilus* CCRC 11223 [Hu et al., 2003]. Finally, *Pseudomonas putida* IFO 12996 [Ogawa and Shimizu, 1994] has the same optimum reaction temperature as found for *S. meliloti* CECT 4114 in the present work. Optimal pH values for the other *L*-N-carbamoylases range from pH 7.4 for *B. kaustophilus* CCRC 11223 to 8.5 for *A. aurescens* DSM 3747, while for N-carbamoyl-*L*-cysteine amidohydrolase from *Pseudomonas* sp. ON-4a it is more alkaline (pH 9.0) [Ohmachi et al., 2004].

The thermostability of *L*-N-carbamoylase from *S. meliloti* was low, with only 20% activity remaining after incubation at 50°C for 30 min. Lower thermostability has been found in *L*-N-carbamoylase of *Pseudomonas* sp. NS 671, with hardly any activity after incubation at 25°C for 60 min [Ishikawa et al., 1993]. On the contrary, *A. aurescens* DSM 3747, *A. xylosoxidans*, *P. putida* IFO 12996 and *B. kaustophilus* CCRC 11223 *L*-N-carbamoylases are very thermostable, with 50% activity remaining after incubation for 2 h at 50°C for the former [Wilms et al., 1999], 70% activity after incubation at 35°C for 30 min for the second [Ogawa et al., 1995], 80% activity remaining after incubation for 30 min at 65°C for *P. putida* IFO 12996 [Ogawa and Shimizu, 1994], and for the latter specific activity increased by 10–20% after incubation at 50°C for 20 min [Hu et al., 2003].

The presence of some divalent metal ions such as Ni^{2+}, Mn^{2+}, Co^{2+} and Fe^{2+} is essential for enzyme activity of *S. meliloti* CECT 4114 *L*-N-carbamoylase. This effect has been reported after reactivation of *L*-N-carbamoylases without initial activity from *B. stearothermophilus* NS 1122A and *Pseudomonas* sp. NS 671 purified in several steps by ammonium sulfate precipitation plus anion exchange chromatography [Ishikawa et al., 1993, 1994]. Likewise, metal ions restore the activity of *L*-N-carbamoylases from *B. kaustophilus* CCRC 11223, *A. xylosoxidans* and *P. putida* IFO 12996 as well as N-carbamoyl-*L*-cysteine amidohydrolase from *Pseudomonas* sp. ON-4a previously treated with the chelating agent EDTA [Hu et al., 2003; Ogawa and Shimizu, 1994; Ogawa et al., 1995; Ohmachi et al., 2004]. There is no information available about divalent metal ion requirement for *L*-N-carbamoylase from *A. aurescens* DSM 3747. Additionally, the three-dimensional structure has recently been resolved for the β-alanine synthase enzyme (N-carbamolyl-β-alanine amidohydrolase) from *S. kluyveri* [Lundgren et al., 2003]. This enzyme has shown a bimetal (zinc) center representing the active site, where the amino acids His^{114} and His^{226} are coordinated with the first zinc molecule and His^{421} and Glu^{160} with the second one. Moreover, the carboxyl group of Asp^{125} serves as a bridging ligand between both zinc ions. These five amino acids present in all *L*-N-carbamoylases (marked with an asterisk in fig. 1), and corresponding to His^{87}, His^{194}, His^{386}, Glu^{133} and Asp^{98} in the *L*-N-carbamoylase sequence from *S. meliloti*, may be involved in the metal-binding capability of the enzyme. The N-carbamoyl-*L*-cysteine amidohydrolase from *Pseudomonas* sp. ON-4a only conserves 4 of the 5 amino acids, showing a serine rather than a histidine in position 397 [Ohmachi et al., 2002].

L-N-carbamoylase from *S. meliloti* showed a broad specificity for N-carbamoyl-*L*-strict amino acids. Of the two groups of *L*-N-carbamoylases classified according to substrate specificity [Ishikawa et al., 1993; Yamashiro et al., 1988], the enzyme studied in this work can be included in the one that shows broad substrate specificity towards N-carbamoyl-*L*-aliphatic and aromatic amino acids. However, enzymes belonging to this group such as *B. kaustophilus* CCRC 11223, *Pseudomonas* sp. NS 671, *B. stearothermophilus* NS 1122A, *A. xylosoxidans* and *P. putida* IFO 12996 have never hydrolysed N-carbamoyl-*L*-tryptophan [Hu et al., 2003; Ishikawa et al., 1993, 1994; Ogawa et al., 1995; Ogawa and Shimizu, 1994]. *L*-N-carbamoylase from *S. meliloti* not only recognizes N-carbamoyl-*L*-tryptophan as a substrate, but also hydrolyses other aromatic and aliphatic N-carbamoyl-*L*-derivatives. Prior to the present paper, only *L*-N-carbamoylase from *A. aurescens* DSM 3747 had hydrolyzed N-carbamoyl-*L*-tryptophan [Wilms et al., 1999], but with very low activity for aliphatic N-carbamoyl-*L*-derivatives. *L*-N-carbamoylase from *S. meliloti* showed activity towards N-carbamoyl-*L*-cysteine substrate, which makes it the first *L*-N-carbamoylase involved in the 'hydantoinase process' that hydrolyses this substrate for *L*-cysteine production. Previous works had only reported *L*-cysteine production from *D,L*-2-amino-Δ^2-thiazoline-4-carboxylic acid (*D,L*-ATC) [Ohmachi et al., 2002; Shiba et al., 2002]. N-Acetyl-*L* and N-formyl-*L*-amino acids were quickly hydrolyzed by *L*-N-carbamoylase from *S. meliloti* CECT 4114. Previous works have described rapid hydrolysis of N-formyl-*L*-amino acids by *L*-N-carbamoylases from *A. xylosoxidans* [Ogawa et al., 1995] and *A. aurescens* DSM 3747 [Wilms et al., 1999], but slow hydrolysis from *P. putida* IFO 12996 [Ogawa and Shimizu, 1994]. Slow hydrolysis of N-Acetyl-*L*-amino acids has been reported by *L*-N-carbamoylases from *A. xylosoxidans* and *P. putida*

IFO 12996 [Ogawa et al., 1995; Ogawa and Shimizu, 1994], and no hydrolysis has been detected from *A. aurescens* DSM 3747 [Wilms et al., 1999]. *L*-N-carbamoylase from *S. meliloti* CECT 4114 did not hydrolyze β-ureidopropionates and ureidosuccinates, unlike *P. putida* IFO 12996, which is the only one that has been reported to do so [Ogawa and Shimizu, 1994].

L-N-carbamoylase from *S. meliloti* was cloned and characterized. The enzyme has proved effective for both aromatic and aliphatic N-carbamoyl-*L*-amino acids. The design of a biological tool to transform racemic mixtures of hydantoins to optically pure *L*-amino acids could be obtained by combining this enzyme with hydantoinase and the recently described hydantoin racemase of this strain [Martinez-Rodriguez et al., 2004]. The creation of this recombinant 'hydantoinase process' as a biocatalyst for the production of *L*-amino acids and its development for industrial application are currently being explored.

Experimental Procedures

General Protocols and Reagents

Standard methods were used for cloning and expression [Ausubel et al., 1995; Sambrook et al., 1989]. Restriction enzymes, T4 DNA ligase and thermostable *Pwo* polymerase for PCR were purchased from Roche Diagnostic S.L. (Barcelona, Spain). N-Carbamoyl-*D,L*-amino butyric acid, N-carbamoyl-*L*-glutamic acid, N-formyl-*L*-methionine, N-acetyl-*L*-methionine, ureidosuccinic acid and 3-ureidopropionic acid were purchased from Sigma-Aldrich (Madrid, Spain). The other N-carbamoyl amino acids used in this work were synthesized according to the literature [Boyd, 1933].

Microbes and Culture Conditions

S. meliloti CECT 4114 and 4857 were used as possible donors of the *L*-N-carbamoylase gene. These strains were cultivated at 30°C for 20 h in Luria-Bertani medium (LB, 1% tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7.2). *E. coli* BL21 [Studier and Moffart, 1986] was used to clone and express the *L*-N-carbamoylase gene.

Cloning and Sequence Analysis of L-*N-Carbamoylase*

S. meliloti genomic DNA was extracted as previously reported [Sobral et al., 1991], and the gene encoding the *L*-N-carbamoylase was amplified by PCR. The primers used were designed based on GenBank sequence accession number NC003047 [Capela et al., 2001; Galibert et al., 2001]. These were SmLcar5 (5′-AATCTAGAGTGACAGGAAAAACGCCATGGCAGCACCTGGCGAGAAT-3′) and SmLcar3 (5′-TTCTGCAGTTAATGATGATGATGATGATGTTCCACGATTTCCGC-CGTTTCGA-3′). The latter included a polyhistidine tag (His_6 tag) before the stop codon. The *Xba*I- and *Pst*I-digested 1,251-bp fragment was purified from agarose gel using QIAquick (Qiagen) and ligated into pBluescript II SK (+) plasmid (pBSK, Stratagene Cloning Systems), to create plasmid pSER35.

Once the fragment had been cloned, it was sequenced using the dye dideoxy nucleotide sequencing method in an ABI 377 DNA Sequencer (Applied Biosystems). Sequencing was carried out at least twice using standard T3 and T7 primers, edited with Microsoft Word 97 and assembled using the CLUSTALW program [Jeanmougin et al., 1998]. The assembled sequences were aligned and compared with all of the amino acid sequence databases available from Internet using Basic Local Alignment Search Tool (BLAST) [Altschul et al., 1990]. Manual comparisons of the sequences were performed with the CLUSTALW program and drawn with ESPript [Gouet et al., 1999]. The percentage of homology between fragments was calculated with BLAST and SEAVIEW [Galtier et al., 1996] programs.

Expression of the L-*N-Carbamoylase*

The transformant in BL21 strain was grown in LB medium supplemented with 100 μg ml^{-1} of ampicillin. A single colony was transferred into 10 ml of LB medium with ampicillin at the above-mentioned concentration in a 100-ml flask. This culture was incubated overnight at 37°C with shaking. In a 2-liter flask 500 ml of LB with the appropriate concentration of ampicillin was inoculated with 5 ml of the overnight culture. After 2 h of incubation at 37°C with vigorous shaking, the OD_{600} of the resulting culture was 0.3–0.5. For expression induction of the *L*-N-carbamoylase gene, isopropyl-β-*D*-thiogalactoside was added to a final concentration of 0.2 m*M* and the culture was continued at 37°C for an additional 4 h. The cells were collected by centrifugation (Beckman JA2-21, 7,000 *g*, 4°C, 10 min), washed twice and resuspended in 50 ml wash buffer (300 m*M* NaCl, 0.02 % NaN_3, 50 m*M* sodium phosphate, pH 7.0). The cell walls were disrupted in ice by sonication using UP 200 S Ultrasonic Processor (Dr. Hielscher GmbH, Germany) for 6 periods of 30 s, pulse mode 0.5 and sonic power 60%. The pellet was precipitated by centrifugation (Beckman JA2–21, 10,000 *g*, 4°C, 20 min) and discarded. The supernatant was applied to a column with TALON™ metal affinity resin (CLONTECH Laboratories Inc.), and then washed 3 or 4 times with wash buffer. After washing, *L*-N-carbamoylase enzyme was eluted with elution buffer (100 m*M* NaCl, 0.02% NaN_3, 50 m*M* imidazole, 2 m*M* Tris, pH 8.0). Before use, the purified enzyme was dialyzed against 0.2 *M* sodium phosphate pH 8.0 and stored at 4°C until use.

Enzyme Assay

Standard enzymatic reaction was carried out with the purified *L*-N-carbamoylase (at a final concentration of 5 μ*M*) together with N-carbamoyl-*L*-methionine as substrate (100 m*M*) dissolved in 200 m*M* sodium phosphate buffer (pH 8.0) in 200 μl reaction volume. The reaction mixture was incubated at 40°C for 20 min and stopped by addition of 4 times the reaction volume of 1% H_3PO_4. After centrifuging, the resulting supernatants were analyzed by HPLC. The HPLC system (Breeze HPLC System, Waters Cromatografia S.A., Barcelona, Spain) equipped with a Symmetry C_{18} column (4.6 × 150 mm, Waters) was used to detect N-carbamoyl-*L*-methionine and *L*-methionine. The mobile phase was methanol/phosphoric acid (20 m*M*) (vol/vol, 5:95), pH 3.2, pumped at a flow rate of 0.75 ml min^{-1} and measured at 210 nm. The specific activity of the enzyme was defined as the amount of enzyme that catalyzed the formation of 1 m*M* of *L*-amino acid at 40°C min^{-1} and mg^{-1} of protein.

Substrate specificity studies were performed with each different N-carbamoyl-amino acid dissolved in 200 m*M* sodium phosphate

buffer (pH 8.0) together with the purified enzyme at the same concentration as described above. Reactions were carried out at 40°C after preincubation of the protein with 2 m*M* $NiCl_2$ and stopped by addition of 1% H_3PO_4. The detection of the different substrates and their corresponding *L*-amino acids was carried out as described above, setting the UV detector to 200 nm for cysteine, glutamic acid, isoleucine, aspartic acid, tyrosine and β-alanine; 205 nm for leucine; 220 nm for tryptophan and 210 nm for the rest of the amino acids. The k_{cat} was defined as the mmol of N-carbamoyl-*L*-amino acid per second and milllimole of enzyme at 40°C.

Molecular Mass Analysis

Size exclusion chromatography-HPLC analysis was performed to estimate the molecular mass of the native enzyme using a non-denatured protein molecular weight marker kit (Sigma Aldrich Quimica S.A., Madrid, Spain). The enzyme was eluted with 0.1 *M* buffers from pH 6.0 to 9.0 at a flow rate of 0.5 ml/min and measured at 280 nm in an HPLC system with a Superdex 200 HR 10/30 column (Amersham Biosciences, Barcelona, Spain). Molecular mass of the monomeric form was estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) by the Laemmli method [Laemmli, 1970] using a low molecular weight marker kit (Amersham Biosciences, Barcelona, Spain).

Protein Characterization

The thermal stability of the *L*-N-carbamoylase enzyme was measured after 30 min of preincubation at temperatures from 20 to 70°C in 0.2 *M* sodium phosphate buffer, pH 8.0. Enzyme assay was then carried out at 40°C for 30 min with the N-carbamoyl-*L*-methionine substrate together with the purified *L*-N-carbamoylase enzyme. To analyze the effect on the enzyme of DTNB, β-mercaptoethanol, DTT, EDTA, $HgCl_2$, $NiCl_2$, $MnCl_2$, $CoCl_2$, $CuCl_2$, $ZnCl_2$, $CaCl_2$, $PbCl_2$, $FeCl_2$, $FeCl_3$, NaCl and KCl, 2 m*M* of each metal and DTNB, and 10 m*M* of β-mercaptoethanol, DTT and EDTA were incubated with the *L*-N-carbamoylase (5 μ*M*) in 0.2 *M* sodium phosphate buffer pH 8.0 (final volume 200 μl) at room temperature for 45 min. The specific activity for the effect of metals was determined by standard enzyme assay.

Nucleotide Sequence Accession Number

The nucleotide sequence of *L*-N-carbamoylase gene of *S. meliloti* (SmehyuC) has been deposited in the GenBank database under accession number AY646850.

Acknowledgments

This work was supported by project PTR-1995-0755-OP and BIO2004-02868 from Ministerio de Educación y Ciencia, Spain. The authors thank Andy Taylor for critical discussion of the manuscript and Pedro Madrid-Romero for technical assistance.

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