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Evaluation of Substrate Promiscuity of an L-Carbamoyl Amino Acid Amidohydrolase from *Geobacillus stearothermophilus* CECT43

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N-carbamoyl-amino-acid amidohydrolase (also known as N-carbamoylase) is the stereospecific enzyme responsible for the chirality of the D- or L-amino acid obtained in the "Hydantoinase Process." This process is based on the dynamic kinetic resolution of D,L-5-monosubstituted hydantoins. In this work, we have demonstrated the capability of a recombinant L-N-carbamoylase from the thermophilic bacterium Geobacillus stearothermophilus CECT43 (BsLcar) to hydrolyze N-acetyl and N-formyl-L-amino acids as well as the known N-carbamoyl-L-amino acids, thus proving its substrate promiscuity. BsLcar showed faster hydrolysis for N-formyl-L-amino acids than for N-carbamoyl and N-acetyl-L-derivatives, with a catalytic efficiency (k_{cat}/K_m) of 8.58×10^5, 1.83×10^4, and 1.78×10^3 (s^{-1} M^{-1}), respectively, for the three precursors of L-methionine. Optimum reaction conditions for BsLcar, using the three N-substituted-L-methionine substrates, were 65°C and pH 7.5. In all three cases, the metal ions Co^{2+}, Mn^{2+}, and Ni^{2+} greatly enhanced BsLcar activity, whereas metal-chelating agents inhibited it, showing that BsLcar is a metalloenzyme. The Co^{2+}-dependent activity profile of the enzyme showed no detectable inhibition at high metal ion concentrations. © 2010 American Institute of Chemical Engineers *Biotechnol. Prog.,* 000: 000–000, 2010

Keywords: L-amino acid production, L-N-carbamoylase, substrate promiscuity, acylase process, hydantoinase process, formylase process

Introduction

Optically pure L-amino acids are biochemically important and of great interest for the chemical industry not only as building blocks of life (proteinogenic amino acids) but also as chiral intermediates for the pharmaceutical, agricultural, and cosmetics industries.[1] They are used as feed and food additives, as intermediates for pharmaceuticals, cosmetics, pesticides, and as chiral synthons in organic synthesis.[2]

Biocatalysis has become an established technology for the industrial manufacture of fine chemicals, and optically pure amino acid production is no exception. One of the biocatalytic tools to obtain optically pure L-amino acids is the "Hydantoinase Process."[3,4] This process is based on the dynamic kinetic resolution of D,L-5-monosubstituted hydantoins using an inexpensive and environment friendly enzymatic method.[5] The chirality of the amino acid obtained depends on the stereospecificity of the last enzyme in the reaction cascade (*N*-carbamoyl-L-amino-acid amidohydrolases, also known as L-*N*-carbamoylases).[6] This enzyme has been found in several microorganisms of the genera *Arthrobacter*,[7] *Alcaligenes*,[8] *Bacillus*,[9] *Blastobacter*,[10] *Flavobacterium*,[11] *Microbacterium*,[12] *Pseudomonas*,[13] and *Sinorhizobium*.[14]

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Enzymes are traditionally considered to be specific catalysts, capable of converting a single substrate to a single product with high efficiency. However, many enzymes are catalytically promiscuous, and they can metabolize structurally distinct substrates or convert a single substrate to multiple products.[15] Enzyme promiscuity can be classified into three types, condition promiscuity, catalytic promiscuity, and substrate promiscuity.[16] Some described that L-*N*-carbamoylases have been able to hydrolyze compounds other than *N*-carbamoyl-L-amino-acids, such as *N*-acetyl or *N*-formyl-L-amino-acids[7–9,13–14] However, this possible substrate promiscuity has not been studied to date.

Our laboratory has recently cloned functionally expressed and purified an L-*N*-carbamoylase from *Geobacillus stearothermophilus* CECT43 (BsLcar) for its crystallization and preliminary crystallographic studies.[17] The aim of this work is to study the substrate promiscuity of the enzyme with different compounds as substrates, and prove that *N*-formyl-amino acids are better substrates for BsLcar than *N*-acetyl and *N*-carbamoyl-amino acids. It may therefore be a better kinetic resolution reaction for L-amino acid production than for the substrate, which gives the name to the enzyme, *N*-carbamoyl-L-amino acid. Reaction conditions, such as pH and temperature, are analysed for the three types of substrates. Additionally, the best metal ion and its optimal concentration for each activity are also determined.

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Material and Methods

General protocols and reagents

Standard methods were used for cloning and expression. Restriction enzymes, T4 DNA ligase, and thermostable *Pwo* polymerase for PCR were purchased from Roche Diagnostic S.L. (Barcelona, Spain). *N*-acetyl D- and L-amino acids were purchased from Sigma-Aldrich (Madrid, Spain) and Avocado Organics (Cymit Quimica, Barcelona, Spain). *N*-formyl-L-methionine and *N*-formyl-L-tyrosine were purchased from Sigma-Aldrich, *N*-formyl-L-valine and *N*-formyl-L-phenylalanine were purchased from Bachem (Cymit Quimica S.L., Barcelona, Spain) and *N*-formyl-L-alanine was purchased from Chem-Impex International. The *N*-carbamoyl amino acids used in this work were synthesized according to the literature.[18]

Microbes and culture conditions

Geobacillus stearothermophilus CECT43 was used as possible donor of the L-N-carbamoylase (Bslcar) gene. This strain was cultivated at 55°C for 20 h in Luria–Bertani medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7.2). *Escherichia coli* BL21[19] was used to clone and express the BsLcar gene.

Cloning, expression, and purification of BsLcar

Cloning, expression, and purification of the recombinant BsLcar have been described in the previous work for its crystallization and preliminary crystallographic studies,[17] and can be summarised as follows. *G. stearothermophilus* CECT43 was chosen as DNA source. The gene was amplified by PCR and cloned in pET-22b(+) plasmid (Novagen) to create the plasmid pJAVI80. The nucleotide sequence of the cloned fragment was exactly the same as that of L-N-carbamoylase from *G. stearothermophilus* NS1122A (GenBank accession No. S67784). L-N-carbamoylase gene was functionally expressed in E. coli BL21. Finally, a one-step purification procedure of the recombinant L-N-carbamoylase fused to the His6-tag was carried out by using immobilized cobalt affinity chromatography. The purified enzyme was dialyzed against 0.1 M sodium phosphate pH 7.5 and stored at 4°C, until use. Protein concentration for the apoenzyme form was calculated based on the extinction coefficients of amino acids.[20]

Enzyme assay

All activity studies were performed with the homodimeric native enzyme. Standard enzymatic reaction was carried out with the purified BsLcar (at final concentrations from 100 to 300 nM) together with *N*-acetyl, *N*-formyl, or *N*-carbamoyl-L-methionine as substrate (15 mM) dissolved in 100 mM sodium phosphate buffer (pH 7.5) in 500 μL reaction volume. The reaction mixture was incubated at 65°C for 30 min and stopped by addition of nine times the reaction volume of 1% H_3PO_4. After centrifuging, the resulting supernatants were analyzed by high performance liquid chromatography (HPLC). The HPLC system (LC2000Plus HPLC System, Jasco, Madrid, Spain) equipped with a Zorbax C_{18} column (3 × 250 mm, Agilent) was used to detect *N*-acetyl, *N*-formyl, and *N*-carbamoyl-L-methionine, and L-methionine. The mobile phase was methanol/phosphoric acid (20 mM, pH 3.2) (vol/vol, 20:80), pumped at a flow rate of 0.50 mL min^{-1} and measured at 200 nm. The specific activity of the enzyme was defined as the amount of enzyme that catalyzed the formation of 1 μmol of L-amino acid at 65°C min^{-1} and mg^{-1} of protein.

Substrate specificity studies were performed with each different *N*-acetyl, *N*-formyl, and *N*-carbamoyl-amino acid dissolved in 100 mM sodium phosphate buffer (pH 7.5) together with the purified enzyme at the same concentration described above. Reactions were carried out at 65°C after preincubation of the protein with 2 mM $CoCl_2$ for 60 min at 4°C and stopped by addition of 1% H_3PO_4. The mobile phase of the different substrates and their corresponding L-amino acids was methanol-phosphoric acid (20 mM, pH 3.2) (5:95 to 30:70 vol/vol, depending on the compound), pumped at a flow rate of 0.50 mL min^{-1}. Compounds were detected with a UV detector at a wavelength of 200 nm. The k_{cat} was defined as the μmol of *N*-acetyl-, *N*-formyl, or *N*-carbamoyl-L-amino acid per second at 65°C.

Protein characterization

Optimal temperature was evaluated from 30 to 80°C in 100 mM sodium phosphate buffer pH 7.5. The thermal stability of the recombinant enzyme was measured after 60 min of preincubation at temperatures from 4 to 75°C in 100 mM sodium phosphate buffer pH 7.5. Studies of pH were assayed in several buffers at pH 5.5 to 10.5 (MES, sodium phosphate, Tris/HCl, borate/HCl and borate/NaOH) at a concentration of 100 mM. Tris/HCl and borate buffers were prepared at 65°C. Enzyme assays, for thermal stability and pH studies, were then carried out at 65°C for 30 min with the *N*-acetyl-, *N*-formyl, and *N*-carbamoyl-L-methionine substrate together with the purified enzyme. To analyse the effect on the enzyme of several chemical agents and metal ions, 2 mM of $HgCl_2$, $NiCl_2$, $MnCl_2$, $CoCl_2$, $CuCl_2$, $ZnCl_2$, $CaCl_2$, $PbCl_2$, $FeCl_2$, $FeCl_3$, NaCl, KCl, and 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), and 10 mM of β–mercaptoethanol, dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), 8-hydroxyquinoline-5-sulfonic acid (HQSA), and iodoacetamide were incubated with the enzyme (1 μM) in 100 mM sodium phosphate buffer pH 7.5 (final volume 500 μL) at 4°C for 60 min. The specific activity for the effect of metals was determined by standard enzyme assay.

Results and Discussion

Recombinant BsLcar was able to hydrolyze *N*-acetyl and *N*-formyl-amino acids in addition to *N*-carbamoyl-amino acids, showing strict L-enantioselectivity for all the three. Additionally, the enzyme showed the fastest amidohydrolase activity on the *N*-formyl-L-amino acid followed by *N*-carbamoyl and *N*-acetyl ones (Figure 1). To explore the optimal conditions of each kinetic resolution for L-amino acid production, physical and biochemical characterizations of the three different reactions were carried out. F1

Temperature and pH dependence in the three kinetic resolutions catalyzed by BsLcar

The amidohydrolase activity of BsLcar for the three different substrates was evaluated at different temperatures, showing maximum activity at 65°C in all cases (Figure 2). In the literature data, on optimal temperature is only available for L-*N*-carbamoylase activity, with values ranging from F2

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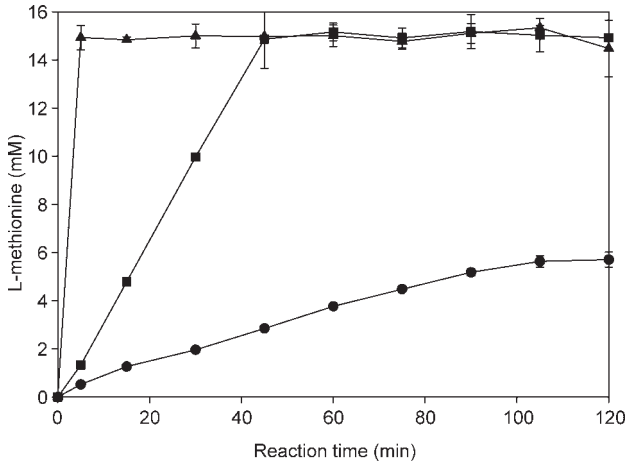

Figure 1. Conversion of three different substrates, *N*-acetyl (●), *N*-formyl (▲), and *N*-carbamoyl-L-methionine (■), into L-methionine by the amidohydrolase activity of BsLcar.

Activity measures were performed using standard enzyme assay from 15 mM of each substrate and in triplicate (see Material and Methods).

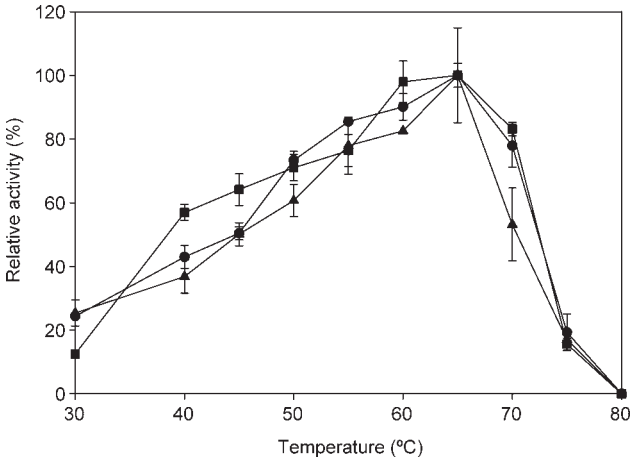

Figure 2. Effect of temperature on BsLcar activity against three different substrates, *N*-acetyl (●), *N*-formyl (▲), and *N*-carbamoyl-L-methionine (■).

Activity measures were performed at different temperatures using standard enzyme assay and in triplicate (see Material and Methods). To compare the effect of temperature on each *N*-substituted amino acid substrate, the maximum activity of BsLcar for each one was denoted as 100% of relative activity.

30°C, for β-ureidopropionase with activity from *A. tumefaciens* C58,[21] to 70°C for L-*N*-carbamoylase of the thermostable microorganisms *G. kaustophilus* CCRC 11223[22] and *G. stearothermophilus* NS 1122A.[23] This latter L-*N*-carbamoylase is the same as BsLcar, but the optimal temperature is higher because the analysis was made in whole cells, while our measures have been taken on purified protein. BsLcar showed maximum activity for *N*-formyl, *N*-acetyl and *N*-carbamoyl-L-methionine in sodium phosphate 100 mM pH 7.5 (Figure 3). Although this is the first time that optimal pH values for *N*-formyl and *N*-acetyl-amino acid amidohydrolase activity (L-*N*-formylase and L-*N*-acetylase) have been studied, they are similar to those described for other L-*N*-carbamoylases. Thus, *B. kaustophilus* CCRC 11223 and *Pseudomonas* sp NS671 L-*N*-carbamoylases have an optimal pH of 7.5,[22,24] which rises to 8.5 for the other L-*N*-carbamoylases,[7–9,13,14,21] with the exception of *N*-carbamoyl-L-cysteine amidohydrolase from *Pseudomonas* sp. ON-4a, which is more alkaline (pH 9.0).[25]

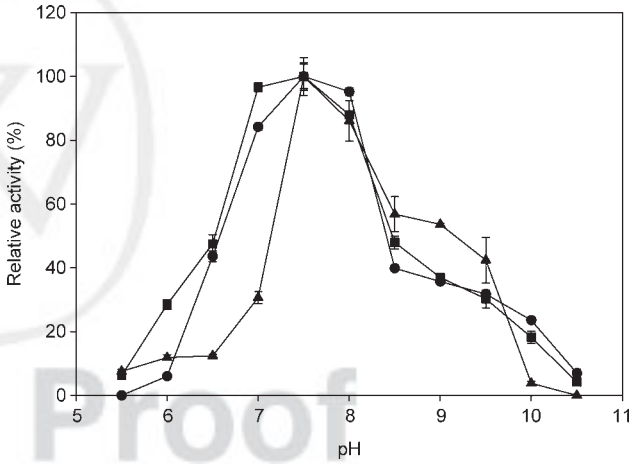

Figure 3. Effect of pH on BsLcar activity against three different substrates, *N*-acetyl (●), *N*-formyl (▲) and *N*-carbamoyl-L-methionine (■).

Activity measures were performed at different pHs at 65°C using standard enzyme assay and in triplicate (see Material and Methods). To compare the effect of pH on each *N*-substituted amino acid substrate, the maximum activity of BsLcar for each one was denoted as 100% of relative activity.

Thermal stability studies performed on BsLcar have shown that maximun activity gradually decreases at temperatures over 65°C for 60 min, with 60% activity remaining after incubation at 70°C for 60 min. β-Ureidopropionase with L-*N*-carbamoylase activity from *P. putida* IFO 12996, which showed high thermostability, retaining 80% of initial activity after incubation at 65°C for 30 min.[13] Other L-*N*-carbamoylases from *A. aurescens* DSM 3747, *A. xylosoxidans*, and *B. kaustophilus* CCRC 11223 have shown 50% activity remaining after incubation for 2 h at 50°C for the first,[7] 70% activity after incubation at 35°C for 30 min for the second,[8] and for the third one specific activity increased by 10–20% after incubation at 50°C for 20 min.[22] However, lower thermostability has been found in L-*N*-carbamoylase from *S. meliloti*, with only 20% activity remaining after incubation at 50°C for 30 min,[15] and in the one from *Pseudomonas* sp. NS 671, with hardly any activity after incubation at 25°C for 60 min.[26]

Effects of chemical agents and metal ions

The importance of metal ions and other chemical agents in the reaction catalyzed by BsLcar was studied using *N*-formyl-L-methionine as substrate (Table 1). The enzymatic function was not affected by the sulfhydryl reagent iodoacetamide and was only slightly affected by DTNB. However, reducing compounds such as β-mercaptoethanol and DTT (both at 10 mM) caused 29 and 41% inhibition, respectively. These results suggest that no cysteine residue is crucial for enzyme activity, although they might have a structural role; on the other hand, the preliminary crystallographic studies of BsLcar do not show any disulfide bridge in its structure.[17] L-*N*-carbamoylases are described as metalloenzymes[6] and BsLcar was not an exception, as the chelating agents EDTA and HQSA, both at 10 mM, decreased its activity drastically to 15 and 13%, respectively. However, when the chelating agent HQSA was removed by dialysis, the enzyme recovered its activity (see Table 1, row "None" in "HQSA pretreated"). This phenomenon probably occurs because of the

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Table 1. Effect of Metal Ions and Chemical Agents on the Activity of BsLcar

	Non HQSA Treated Enzyme		HQSA Pretreated Enzyme	
Compound	Specific Activity (U mg^{-1})	Relative Activity (%)	Specific Activity (U mg^{-1})	Relative Activity (%)
None	33.34 ± 2.56	100 ± 7.67	40.20 ± 5.90	120.55 ± 17.69
Co^{2+}	221.37 ± 25.51	663.89 ± 76.50	179.03 ± 37.14	536.91 ± 111.38
Ni^{2+}	114.02 ± 8.36	341.94 ± 25.07	111.35 ± 15.28	333.96 ± 45.83
Mn^{2+}	95.67 ± 7.84	286.93 ± 23.52	65.69 ± 9.44	197.00 ± 28.31
Mg^{2+}	31.68 ± 1.55	95.01 ± 4.66	30.45 ± 4.23	91.32 ± 12.68
Zn^{2+}	38.66 ± 0.52	115.95 ± 1.55	33.32 ± 4.65	99.93 ± 13.94
Cu^{2+}	45.75 ± 2.49	137.19 ± 7.46	39.37 ± 0.45	118.07 ± 1.36
Ca^{2+}	31.84 ± 1.80	95.50 ± 5.39	35.37 ± 1.88	106.07 ± 5.62
Hg^{2+}	8.69 ± 2.99	26.06 ± 8.98	11.40 ± 1.83	34.17 ± 5.48
Fe^{2+}	1.01 ± 0.12	3.04 ± 0.36	1.11 ± 0.70	3.34 ± 2.11
Pb^{2+}	25.22 ± 1.46	75.63 ± 4.38	27.07 ± 0.80	81.18 ± 2.41
Fe^{3+}	14.50 ± 5.20	43.48 ± 15.60	20.17 ± 1.98	60.49 ± 5.94
K^{+}	39.59 ± 4.73	118.73 ± 14.19	29.23 ± 4.03	87.65 ± 12.08
Cs^{+}	29.29 ± 5.10	87.83 ± 15.29	30.61 ± 2.05	91.80 ± 6.14
Na^{+}	29.15 ± 3.63	87.41 ± 10.89	34.55 ± 4.08	103.62 ± 12.24
Li^{+}	31.48 ± 3.51	94.41 ± 10.54	24.38 ± 3.04	73.10 ± 9.12
EDTA	5.12 ± 3.07	15.34 ± 9.20	4.82 ± 2.89	14.44 ± 8.66
HQSA	4.44 ± 2.41	13.33 ± 7.22	4.18 ± 2.27	12.55 ± 6.80
DTNB	29.17 ± 4.77	87.49 ± 14.29	25.51 ± 3.26	76.51 ± 9.77
DTT	19.58 ± 3.99	58.74 ± 11.97	18.23 ± 1.25	54.68 ± 3.74
B-mercaptoethanol	23.71 ± 0.12	71.11 ± 0.36	28.02 ± 159	84.04 ± 4.78
Iodoacetamide	38.80 ± 1.33	116.36 ± 3.98	37.24 ± 6.11	111.67 ± 18.31

The metal ion chloride salts, reducing, sulphydryl and chelating reagents, were preincubated with the enzyme. The restoration of BsLcar activity by metal ions after treatment with the HQSA chelating agent was also studied. The enzyme was incubated overnight with 10 mM of HQSA at 4°C. The chelating agent was removed by dialysis in four stages at 4-hour intervals, all at 4°C. Activity assays were carried out in triplicate as described in Material and Methods with *N*-formyl-L-methionine as substrate.

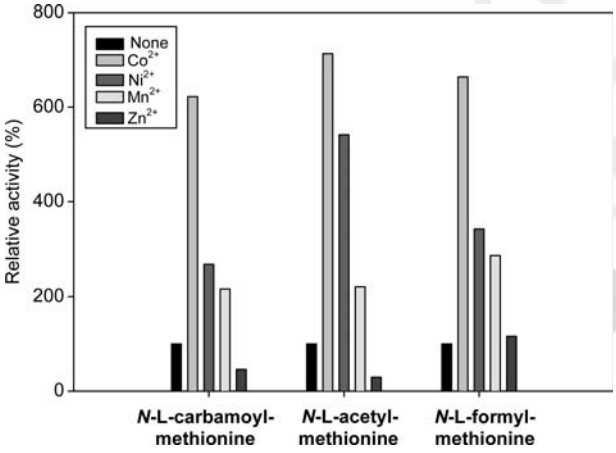

Figure 4. Variation of BsLcar activity for the hydrolysis of *N*-carbamoyl, *N*-acetyl and *N*-formyl-L-methionine after incubation at 4°C for 60 min with Co^{2+}, Ni^{2+}, Mn^{2+}, and Zn^{2+}.

To compare the effect of metal ions on each *N*-substituted amino acid substrate, the maximum activity of BsLcar for each one was denoted as 100% of relative activity.

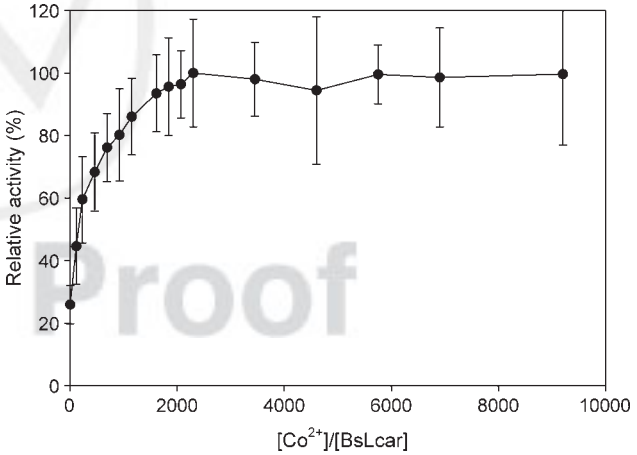

Figure 5. Effect of Co^{2+} metal ion on BsLcar activity.

The reaction mixture contained 100 mM sodium phosphate buffer (pH 7.5), 10 mM *N*-formyl-L-methionine substrate, 100 nM of BsLcar, and several different Co^{2+} concentrations. Data are the means of three independent experiments and are shown as percentages of the enzyme activity at different Co^{2+}/BsLcar ratios.

presence of trace metal ions in the dialysis buffer, which may act as a cofactor.

The activation or inhibition effect on BsLcar of different metal ions was also assayed, evaluating its activity in the presence of 2 mM of each compound, using both the non-HQSA-treated and HQSA-treated forms (Table 1). Incubation of the enzyme with several divalent metal ions, such as Fe^{2+} caused total inhibition. Incubation with Hg^{2+} and Fe^{3+} resulted in strong inhibition, while Pb^{2+}, Cs^{2+}, and Na^{+} caused only slight inhibition. However, metal ions such as Co^{2+}, Ni^{2+}, and Mn^{2+} greatly enhanced activity. The same results were obtained for both non-HQSA-treated and HQSA-treated forms of the enzyme. Consequently, it was decided to incubate BsLcar with the divalent metal ion Co^{2+} before activity assays.

The enhancer effect of metal ions on L-*N*-carbamoylase activity has been previously reported, with the reactivation of L-*N*-carbamoylases without initial activity from *B. stearothermophilus* NS 1122A and *Pseudomonas* sp. NS 671 purified in several steps by ammonium sulphate precipitation plus anion exchange chromatography.[23,26] Likewise, metal ions restore the activity of L-*N*-carbamoylases from *B. kaustophilus* CCRC 11223, *A. xylosoxidans*, *S. meliloti* CECT 4114, 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.[8,13,14,22,25]

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Table 2. Kinetic Parameters of BsLcar Against *N*-acetyl, *N*-formyl, and *N*-carbamoyl-Amino Acids

Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})
N-Carbamoyl-L-methionine	2.90 ± 0.46	5.32 ± 0.04	1834.48 ± 277.19
N-Carbamoyl-L-alanine	4.19 ± 0.54	48.96 ± 2.22	11684.96 ± 976.11
N-Carbamoyl-L-valine	3.29 ± 0.62	0.84 ± 0.04	255.32 ± 35.96
N-Carbamoyl-L-phenylalanine	5.82 ± 0.45	0.16 ± 0.00	27.49 ± 2.13
N-Carbamoyl-L-tyrosine	12.99 ± 2.43	0.18 ± 0.00	13.86 ± 2.59
N-Acetyl-L-methionine	12.66 ± 1.99	2.26 ± 0.14	178.52 ± 17.00
N-Acetyl-L-alanine	36.12 ± 4.23	27.86 ± 1.60	771.32 ± 46.03
N-Acetyl-L-valine	13.92 ± 1.14	0.08 ± 0.00	5.96 ± 0.49
N-Acetyl-L-phenylalanine	12.90 ± 2.50	0.01± 0.00	0.39 ± 0.08
N-Acetyl-L-tyrosine	115.99 ± 17.25	0.08 ± 0.00	0.69 ± 0.10
N-Formyl-L-methionine	9.77 ± 0.95	839.06 ± 32.34	85881.27 ±5040.66
N-Formyl-L-alanine	5.00 ± 0.94	293.48 ± 20.36	58696.00 ± 6962.85
N-Formyl-L-valine	6.75 ± 0.81	27.26 ± 0.99	4038.52 ± 337.96
N-Formyl-L-phenylalanine	13.82 ± 1.43	12.12 ± 0.56	876.99 ± 50.22
N-Formyl-L-tyrosine	23.78 ± 2.70	1.46 ± 0.08	61.40 ± 3.61

Diferent metal ions have been suggested as cofactors for L-*N*-carbamoylases, namely Zn^{2+}, Ni^{2+}, Co^{2+}, Mn^{2+}, Mg^{2+}, and Fe^{2+}.[6] As Table 1 shows Co^{2+} was the best metal ion for BsLcar regarding L-*N*-formylase activity, with twice the activity produced after incubation with Ni^{2+} or Mn^{2+}. To evaluate whether different metal ions modify the catalytic activity of the enzyme, the L-*N*-formylase, L-*N*-acetylase, and L-*N*-carbamoylase activities of BsLcar were studied after incubation with several metal ions (Figure 4). For all three activities of BsLcar the best cofactor was again Co^{2+}, followed by Ni^{2+}, Mn^{2+}, and Zn^{2+}. The best metal ion/protein ratio was found to be 2000:1 when Co^{2+} was present for at least 60 min at 4°C (Figure 5). Under these conditions, maximum activity was maintained and no inhibition was detected.

Substrate promiscuity of BsLcar. Different kinetic resolutions for L-amino acids production

The ability of purified BsLcar to hydrolyze different substrates was examined. To this end, the kinetic parameters K_m, k_{cat}, and k_{cat}/K_m were obtained from hyperbolic saturation curves by least-squares fitting of the data to the Michaelis–Menten equation. Reactions were carried out with different concentrations of substrates at 65°C after preincubation of the enzyme with $CoCl_2$. Substrate promiscuity of BsLcar allowing L-amino acids production by several kinetic resolutions is detailed in Table 2. The enzyme showed efficient amidohydrolase activity for both aliphatic and aromatic *N*-formyl, *N*-acetyl and *N*-carbamoyl-L-specific derivatives, whereas it did not hydrolyse the D-amino acids precursors. The fastest hydrolysable type of substrate was the *N*-formyl-L-amino acid, followed by the *N*-carbamoyl and *N*-acetyl ones. In all three, BsLcar showed better hydrolysis capability for aliphatic than for aromatic substrates.

Slow hydrolysis of *N*-acetyl-L-amino acids by L-*N*-carbamoylases from *A. xylosoxidans* and *P. putida* IFO 12996 has been reported.[8,13] The L-*N*-acetylase activity for *S. meliloti* L-*N*-carbamoylase is slightly faster, showing higher catalytic efficiency than for the hydrolysis of *N*-formyl-L-amino acids.[14] Our laboratory has recently characterized *N*-succinylamino acid racemase (NSAAR) with *N*-acylamino acid racemase (NAAAR) activity from *Geobacillus kaustophilus* CECT4264.[27] This enzyme, together with BsLcar with L-*N*-acetylase activity, may allow us to obtain the dynamic kinetic resolution of optically pure L-amino acids from racemic mixtures of acetylamino acids.

This is the first study to determine the substrate specificity of an L-*N*-carbamoylase toward different *N*-formyl and *N*-acetyl-L-amino acids. Previous works have described the hydrolysis of *N*-formyl-L-amino acids by L-*N*-carbamoylases from *A. aurescens* DSM 3747,[7] *A. xylosoxidans*,[8] *P. putida* IFO 12996,[13] and *S. meliloti*.[14] However, our work demonstrates that the affinity (K_m values) and particularly the velocity of the hydrolysis, are superior for aliphatic derivatives. Additionally, it is the first time that an L-*N*-carbamoylase has presented better catalytic efficiency for *N*-formyl-L-amino acids than for *N*-carbamoyl-ones. These results may indicate a new way to produce optically pure L-amino acids from racemic mixtures of *N*-formyl-amino acids together with a previously described *N*-succinylamino acid racemase from *Amycolatopsis* sp. TS-1-60 with *N*-formylamino acid racemase activity.[28]

This study has demonstrated the substrate promiscuity of BsLcar. The enzyme was able to hydrolyse *N*-acetyl, *N*-formyl, and *N*-carbamoyl-L-amino acids at the same optimal pH and temperature (7.5 and 65°C). BsLcar has shown the best kinetic resolution for L-amino acids production when *N*-formyl-L-derivatives were used. To our knowledge, this is the first work to report different kinetic resolutions on the promiscuity of L-*N*-carbamoylase for compounds other than its namesake, *N*-carbamoyl-L-amino-acid. This BsLcar substrate promiscuity could open new routes for L-amino acids production, such as the "Formylase Process," using its L-*N*-formylase activity. Moreover, the L-*N*-acylase activity of BsLcar could be used to produce L-amino acids by the "Acylase Process." In both processes, a dynamic kinetic resolution from racemic mixtures of substrates could be obtained after combining BsLcar with an *N*-succinyl-amino acid racemase with demonstrated *N*-acetyl and *N*-formyl-amino acid racemase activity, respectively.[27,28]

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