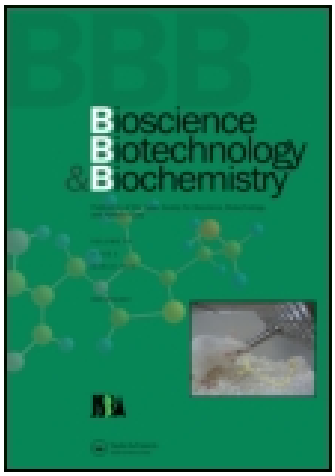
This article was downloaded by: [North West University]
On: 19 December 2014, At: 16:11
Publisher: Taylor & Francis
Informa Ltd Registered in England and Wales Registered Number: 1072954 Registered office: Mortimer House, 37-41 Mortimer Street, London W1T 3JH, UK



Bioscience, Biotechnology, and Biochemistry
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/tbbb20

N-Carbamyl-L-Amino Acid Amidohydrolase of Pseudomonas sp. Strain NS671: Purification and Some Properties of the Enzyme Expressed in Escherichia coli

Takahiro Ishikawa[a], Ken Watabe[a], Yukuo Mukohara[a] & Hiroaki Nakamura[a]
[a] Odawara Research Center, Nippon Soda Co., Ltd., 345 Takada, Odawara, Kanagawa 250-02, Japan
Published online: 12 Jun 2014.

To cite this article: Takahiro Ishikawa, Ken Watabe, Yukuo Mukohara & Hiroaki Nakamura (1996) N-Carbamyl-L-Amino Acid Amidohydrolase of Pseudomonas sp. Strain NS671: Purification and Some Properties of the Enzyme Expressed in Escherichia coli, Bioscience, Biotechnology, and Biochemistry, 60:4, 612-615, DOI: 10.1271/bbb.60.612

To link to this article: http://dx.doi.org/10.1271/bbb.60.612

PLEASE SCROLL DOWN FOR ARTICLE

Taylor & Francis makes every effort to ensure the accuracy of all the information (the "Content") contained in the publications on our platform. However, Taylor & Francis, our agents, and our licensors make no representations or warranties whatsoever as to the accuracy, completeness, or suitability for any purpose of the Content. Any opinions and views expressed in this publication are the opinions and views of the authors, and are not the views of or endorsed by Taylor & Francis. The accuracy of the Content should not be relied upon and should be independently verified with primary sources of information. Taylor and Francis shall not be liable for any losses, actions, claims, proceedings, demands, costs, expenses, damages, and other liabilities whatsoever or howsoever caused arising directly or indirectly in connection with, in relation to or arising out of the use of the Content.

This article may be used for research, teaching, and private study purposes. Any substantial or systematic reproduction, redistribution, reselling, loan, sub-licensing, systematic supply, or distribution in any form to anyone is expressly forbidden. Terms & Conditions of access and use can be found at http://www.tandfonline.com/page/terms-and-conditions

Biosci. Biotech. Biochem., **60** (4), 612–615, 1996

N-Carbamyl-L-Amino Acid Amidohydrolase of *Pseudomonas* sp. Strain NS671: Purification and Some Properties of the Enzyme Expressed in *Escherichia coli*

Takahiro ISHIKAWA, Ken WATABE, Yukuo MUKOHARA, and Hiroaki NAKAMURA

Odawara Research Center, Nippon Soda Co., Ltd., 345 Takada, Odawara, Kanagawa 250-02, Japan

Received October 5, 1995

An *N*-carbamyl-L-amino acid amidohydrolase was purified from cells of *Escherichia coli* in which the gene for *N*-carbamyl-L-amino acid amidohydrolase of *Pseudomonas* sp. strain NS671 was expressed. The purified enzyme was homogeneous by the criterion of SDS–polyacrylamide gel electrophoresis. The results of gel filtration chromatography and SDS–polyacrylamide gel electrophoresis suggested that the enzyme was a dimeric protein with 45-kDa identical subunits. The enzyme required Mn^{2+} ion (above 1 mM) for the activity. The optimal pH and temperature were 7.5 and around 40°C, respectively, with *N*-carbamyl-L-methionine as the substrate. The enzyme activity was inhibited by ATP and was lost completely with *p*-chloromercuribenzoate (1 mM). The enzyme was strictly L-specific and showed a broad substrate specificity for *N*-carbamyl-L-α-amino acids.

Key words: *N*-carbamyl-L-amino acid amidohydrolase; *Pseudomonas* sp. strain NS671

Downloaded by [North West University] at 16:11 19 December 2014

N-Carbamyl-L-amino acid amidohydrolases, by which *N*-carbamyl-L-amino acids are hydrolyzed to L-amino acids, have been reported in some microorganisms, but these enzymes have not been highly purified from any of the sources.[1] Recently, β-ureidopropionase with *N*-carbamyl-L-α-amino acid amidohydrolase activity was purified to homogeneity and characterized.[2]

We have reported the stereospecific conversion of racemic 5-substituted hydantoins to the corresponding L-amino acids by *Pseudomonas* sp. strain NS671,[3] and the cloning of the genes for the three enzymes (hydantoin racemase, hydantoinase, and *N*-carbamyl-L-amino acid amidohydrolase) involved in the conversion from the native plasmid of strain NS671.[4,5] The cloning and expression of the gene, *hyuC*, for *N*-carbamyl-L-amino acid amidohydrolase in *Escherichia coli* (*E. coli*) enabled us to purify the gene product with relative ease.

This paper deals with the purification and characterization of the *hyuC* product, *N*-carbamyl-L-amino acid amidohydrolase of strain NS671, expressed in *E. coli*.

Materials and Methods

Microorganisms and plasmid. Plasmid pDST38[4] is a derivative of pUC18 containing the gene for *N*-carbamyl-L-amino acid amidohydrolase of *Pseudomonas* sp. strain NS671. *E. coli* JM103 carrying pDST38 was used for purification of the *N*-carbamyl-L-amino acid amidohydrolase.

Chemicals. *N*-Carbamyl-methionine was prepared from methionine, and other chemicals were the best available commercial products.[3]

Enzyme assay. The activity of *N*-carbamyl-L-amino acid amidohydrolase was estimated by measuring the amount of L-methionine produced from *N*-carbamyl-L-methionine. Details of the standard assay method are as follows: the reaction mixture (400 μl) containing 13 mM of *N*-carbamyl-L-methionine, 1 mM of $MnCl_2$, 100 mM of Tris H_3PO_4 buffer (pH 7.8), and a sample of enzyme was incubated at 30°C for 60 min unless otherwise stated. One unit of enzyme activity was defined as the activity that catalyzes the formation of 1 μmol of L-methionine from *N*-carbamyl-L-methionine per min under these conditions.

Purification of the N-carbamyl-L-amino acid amidohydrolase from E. coli JM103 carrying pDST38. *E. coli* JM103 carrying pDST38 was grown at 30°C in 3 liters of M9 minimum salt medium[6] containing 10 g/liter of glycerol, 20 g/liter of yeast extract, 1.2 g/liter of $MgSO_4 \cdot 7H_2O$, 0.2 g/liter of $MnCl_2 \cdot 4H_2O$, 75 mg/liter of $CaCl_2 \cdot 2H_2O$, and 50 mg/liter of ampicillin with a 5-liter jar fermentor (Mituwa Rikagaku Kogyo). Isopropyl-1-thio-β-D-galactopyranoside (final concentration, 1 mM) was added to the culture at an A_{600} of 10, and cultivation was continued for another 3.5 h. The cells were harvested by centrifugation and stored at −20°C.

All the following purification steps were done at 0 to 4°C. The frozen cells (97 g as wet weight) were suspended in 1000 ml of 25 mM sodium phosphate buffer (pH 7.5), and disrupted with a Gaulin Homogenizer Model 15 MR (APV Gaulin Inc., U.S.A.) by two passages at 8000 lb/in^2. The resultant suspension was centrifuged at 12,000 × *g* for 30 min. The supernatant was fractionated by stepwise addition of solid ammonium sulfate. The precipitate obtained from 16 to 60% saturation was dissolved in 200 ml of 20 mM sodium phosphate buffer (pH 7.4) containing 0.4 M ammonium sulfate and was put on a phenyl-Sepharose CL-4B (Pharmacia LKB, Sweden) column (4.5 by 40 cm) equilibrated previously with the same buffer. The column was first washed with the same buffer, then 20 mM sodium phosphate buffer (pH 7.4) containing 0.2 M ammonium sulfate. Since the activity was partly detected in eluate with 0.2 M ammonium sulfate, elution was done with H_2O. Active fractions eluted with H_2O were put on a DEAE-cellulose DE-52 (Whatman, England) column (2.2 by 22 cm) equilibrated previously with 50 mM Tris H_3PO_4 buffer (pH 7.8). After the column was washed thoroughly, elution was done with a linear gradient of 0.05 to 1 M Tris H_3PO_4 buffer. Active fractions, found in eluate with about 0.2–0.3 M Tris H_3PO_4 buffer, were concentrated by ultrafiltration and mixed with ammonium sulfate (final, 0.4 M). The supernatant was again put on a phenyl-Sepharose CL-4B column (2.2 by 22 cm) equilibrated previously with 50 mM sodium phosphate buffer (pH 7.4) containing 0.4 M ammonium sulfate. After the column was washed thoroughly, elution was done with a linear gradient of 0.4 to 0 M ammonium sulfate. Active fractions, found in eluate with 0.24–0.15 M ammonium sulfate, were concentrated by ultrafiltration. The concentrate was further purified by gel filtration with a Sephadex G-100 Superfine (Pharmacia LKB) column (2.6 by 86.5 cm) equilibrated with 0.1 M sodium phosphate buffer (pH 7.4) containing 0.2 M NaCl. Active fractions were pooled and concentrated by ammonium sulfate precipitation (80% saturation). The precipitate was dissolved in 8 ml of 20 mM sodium phosphate buffer (pH 7.4). After centrifugation at 18,000 × *g* for 10 min, the supernatant was used as purified *N*-carbamyl-L-amino acid amidohydrolase.

Analytical methods. Methionine and *N*-carbamyl-amino acid in the reaction mixture were detected by thin-layer chromatography[3] and were measured by high pressure liquid chromatography essentially the same as described previously.[3] The configurations of amino acids were identified by the ligand exchange method.[3]

Abbreviation: *E. coli*, *Escherichia coli*.

NII-Electronic Library Service

Downloaded by [North West University] at 16:11 19 December 2014

The molecular mass of a denatured *N*-carbamyl-L-amino acid amidohydrolase was estimated by SDS-polyacrylamide gel electrophoresis.[7] The molecular mass of the native *N*-carbamyl-L-amino acid amidohydrolase was measured by gel filtration with a Sephadex G-200 superfine (Pharmacia LKB) column (3.2 by 85 cm) equilibrated previously with 0.1 M sodium phosphate buffer (pH 7.4) containing 0.2 M NaCl. For molecular mass estimation, catalase (232 kDa), aldolase (158 kDa), bovine serum albumin (67.0 kDa), ovalbumin (43.0 kDa), chymotrypsinogen A (25.0 kDa), and ribonuclease A (13.7 kDa) (gel filtration calibration kit; Pharmacia LKB) were used as standards.

The amount of protein was measured by the method described by Lowry *et al.*[8] with bovine serum albumin used as a standard. The isoelectric point was estimated by using a Model 111 Mini IEF Cell (Bio-Rad Laboratories). For N-terminal sequence analysis, the purified enzyme was put on a pulsed-liquid-phase sequencer (477A; Applied Biosystems) with an on-line phenylthiohydantoin analyzer (120A; Applied Biosystems).

Results

Purification of the N-carbamyl-L-amino acid amidohydrolase

The *N*-carbamyl-L-amino acid amidohydrolase was purified from a crude extract of cells of *E. coli* JM103 carrying pDST38[4] in which the gene was expressed by addition of isopropyl-1-thio-β-D-galactopyranoside (see Materials and Methods). The results of purification are shown in Table I.

The resulting *N*-carbamyl-L-amino acid amidohydrolase showed a single band on a 10% SDS–polyacrylamide gel (Fig. 1). The molecular mass of the band was estimated to be 45 kDa, and it was in good agreement with the calculated molecular mass of the *hyuC* gene product (45.7 kDa).[4] By gel filtration chromatography with Sephadex G-200 superfine, the molecular mass of the enzyme was estimated to be approximately 109 kDa (Fig. 2). These findings suggest that the *N*-carbamyl-L-amino acid amidohydrolase is a dimer consisting of an identical subunit.

The N-terminal amino acid sequence of the purified enzyme was analyzed and found to be Met-Lys-Thr-Val-Thr-Ile-Ser-Lys-Glu-X-Leu- (where X indicates an unidentified residue). The N-terminal amino acid sequence agreed with that of the *hyuC* product predicted from the DNA sequence.[4]

The specific activity expressed as μmol/min/mg protein was 21.5 at pH 7.5 and 42°C, and the isoelectric point was 4.8.

Activation of the enzyme by metal ions

When the purified *N*-carbamyl-L-amino acid amidohydrolase was incubated with *N*-carbamyl-L-methionine, the production of L-methionine was not observed. So the activation effects of metal ions were examined. As is shown in Table II, Co^{2+}, Mn^{2+}, or Ni^{2+} ion activated the enzyme among the tested metal ions. The addition of Mn^{2+} ion at above 1 mM resulted in the efficient production of L-

Table I. Purification of *N*-Carbamyl-L-Amino Acid Amidohydrolase

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)
Crude extract	48,800	ND	--	
Ammonium sulfate	44,200	2,040	0.046	(100)
Phenyl-Sepharose (I)	5,850	1,090	0.186	53.4
DEAE-Cellulose	836	ND	--	--
Phenyl-Sepharose (II)	88.8	366	4.12	17.9
Sephadex G-100	29.7	229	7.71	11.2
Ammonium sulfate	17.1	188	11.0	9.2

Yield based on total activity of ammonium sulfate fractionation step. ND, not determined.

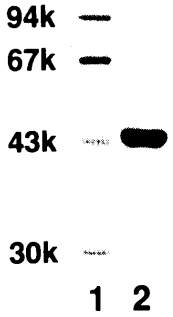


Fig. 1. SDS Polyacrylamide Gel Electrophoresis Analysis of Purified *N*-Carbamyl-L-Amino Acid Amidohydrolase.

A purified *N*-carbamyl-L-amino acid amidohydrolase sample was treated with SDS at 100 C for 5 min in the presence of 2-mercaptoethanol and was analyzed on 10% polyacrylamide gel. Lane 1, molecular mass markers; lane 2, purified *N*-carbamyl-L-Amino acid amidohydrolase.

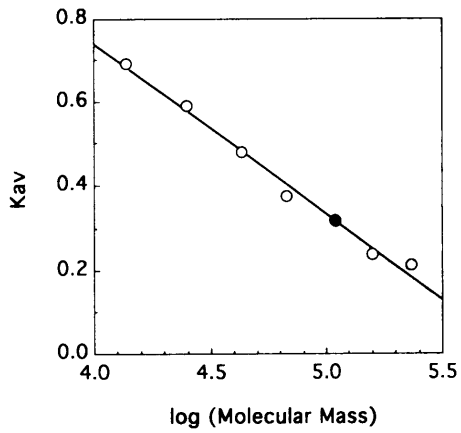


Fig. 2. Measurement of the Relative Molecular Mass of *N*-Carbamyl-L-Amino Acid Amidohydrolase by Gel Filtration Chromatography with Sephadex G-200 superfine.

N-Carbamyl-L-amino acid amidohydrolase and standard proteins were chromatographed individually over Sephadex G-200 superfine gel as described in Materials and Methods. K_{av} is defined as $(V_e - V_0)/(V_t - V_0)$ with V_e, V_t, V_0 being the elution volume, the column volume, and the void volume, respectively. Symbols: ●, *N*-carbamyl-L-amino acid amidohydrolase; ○, standard proteins.

Table II. Effects of Metal Ions on the Activity of *N*-Carbamyl-L-Amino Acid Amidohydrolase

Metal ions (mM)		L-Methionine produced (mM)
$CoSO_4 \cdot 7H_2O$	0.5	1.0
	5.0	1.7
$MnSO_4 \cdot 4\text{–}6H_2O$	0.5	2.3
	5.0	8.4
$NiCl_2$	0.5	0.7
	5.0	1.3
$AlCl_3$, $BaCl_2$, $CaCl_2$, $CuSO_4$, $FeSO_4$, $MgSO_4$, Na_2MoO_4, NaCl, $SnCl_2$, $ZnSO_4$		not detected

The reaction mixture contained 26 mM of *N*-carbamyl-L-methionine, 0.5 or 5.0 mM of metal ion, and the purified enzyme in 0.1 M sodium phosphate buffer (pH 7.5). The reactions were done at 42 C for 60 min.

NII-Electronic Library Service

methionine (Fig. 3).

Effects of pH on the enzyme activity

Variation of the enzyme activity with pH was observed. The profile of the activity is illustrated in Fig. 4. The optimal pH for L-methionine production was found to be 7.5.

Effects of temperature on the enzyme activity

The effects of temperature on the enzyme activity at pH 7.5 were examined. As can be seen from Fig. 5, the optimal temperature for L-methionine production was around 40°C.

The thermal stability of the enzyme was found to be low because the remaining activity was scarcely detected after incubation of the enzyme solution (pH 7.5) at 25°C for 60 min.

Inhibition of the enzyme activity by ATP and p-chloromercuribenzoate

We previously reported that the hydrolysis of *N*-carbamyl-L-methionine was blocked in the presence of high concentrations (5–10 mM) of ATP when the crude extract of strain NS671 was used as an enzyme source.[3] So the effect of ATP on the enzyme activity was examined by using the purified *N*-carbamyl-L-amino acid amidohydrolase. It can be seen from Fig. 6 that the enzyme activity was in-

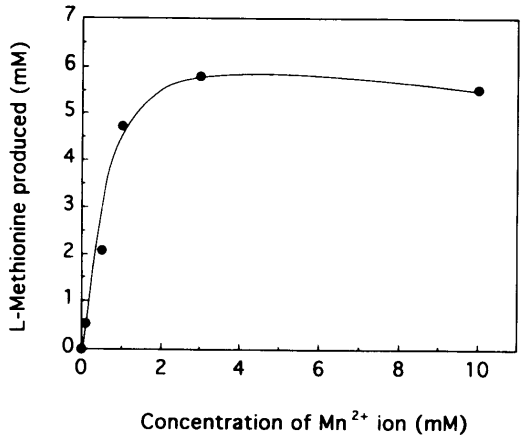


Fig. 3. Effects of Mn^{2+} Ion on the Activity of *N*-Carbamyl-L-Amino Acid Amidohydrolase.

The reaction mixture contained 26 mM of *N*-carbamyl-L-methionine, Mn^{2+} ion at the indicated concentrations, and the purified enzyme in 0.1 M sodium phosphate buffer (pH 7.5). The reactions were done as described in Table II.

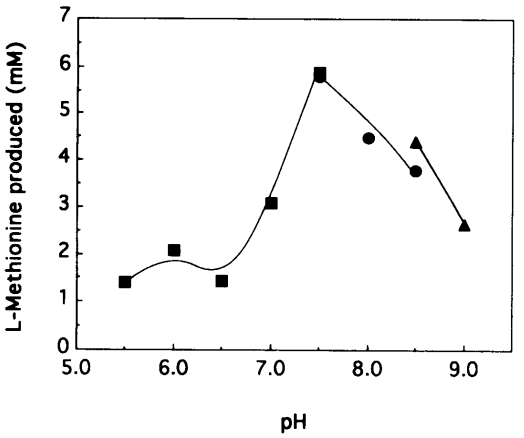


Fig. 4. Effects of pH on the Activity of *N*-Carbamyl-L-Amino Acid Amidohydrolase.

The reaction mixture contained 26 mM of *N*-carbamyl-L-methionine, 0.5 mM of Mn^{2+} ion, and the purified enzyme in the indicated buffer. The reactions were done at 30°C for 60 min in the following buffers: ■, 0.1 M sodium phosphate; ●, 0.1 M Tris HCl; ▲, 0.2 M NH_4Cl NH_4OH.

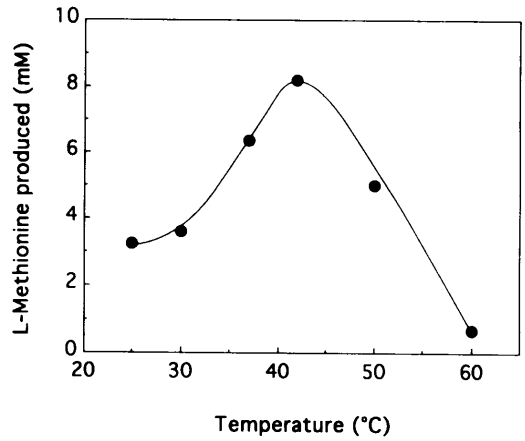


Fig. 5. Effects of Temperature on the Activity of *N*-Carbamyl-L-Amino Acid Amidohydrolase.

The reaction mixture contained 26 mM of *N*-carbamyl-L-methionine, 0.5 mM of Mn^{2+} ion, and the purified enzyme in 0.1 M sodium phosphate buffer (pH 7.5). The reactions were done at the indicated temperatures for 60 min.

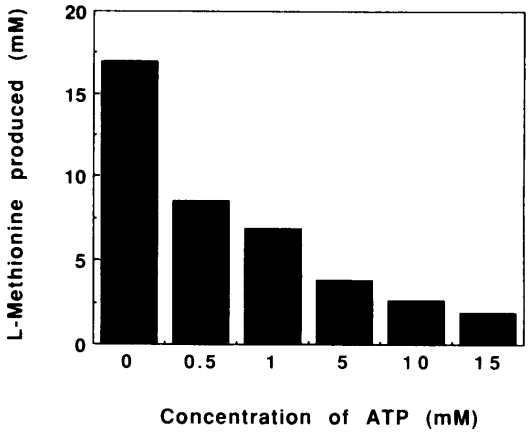


Fig. 6. Inhibition of *N*-Carbamyl-L-Amino Acid Amidohydrolase Activity by ATP.

The reaction mixture contained 26 mM of *N*-carbamyl-L-methionine, 0.5 mM of Mn^{2+} ion, ATP at the indicated concentrations, and the purified enzyme in 0.1 M sodium phosphate buffer (pH 7.5). The reactions were done at 30 C for 60 min.

Table III. Substrate Specificity of *N*-Carbamyl-L-Amino Acid Amidohydrolase

Substrates	Products	Yield (mM)
N-Carbamyl-L-methionine	L-Methionine	17.7
N-Carbamyl-DL-methionine	L-Methionine	16.7
N-Carbamyl-DL-alanine	L-Alanine	18.1
N-Carbamyl-DL-valine	L-Valine	18.9
N-Carbamyl-L-leucine	L-Leucine	20.9
N-Carbamyl-L-isoleucine	L-Isoleucine	17.1
N-Carbamyl-DL-phenylalanine	L-Phenylalanine	16.7
N-Carbamyl-L-tyrosine	L-Tyrosine	10.7
N-Carbamyl-β-alanine		0

The reaction mixture contained 25 mM (based on L-form) of *N*-carbamyl-amino acid, 0.5 mM of Mn^{2+} ion, and 0.14 mg/ml of the purified enzyme in 0.1 M sodium phosphate buffer (pH 7.6). The reactions were done at 30°C for 30 min. For tyrosine, the yield was calculated from the residual amount of *N*-carbamyl-L-tyrosine because L-tyrosine was precipitated from the reaction mixture.

Downloaded by [North West University] at 16:11 19 December 2014

NII-Electronic Library Service

hibited by ATP.

Effects of *p*-chloromercuribenzoate, an SH-reagent, on enzyme activity were also examined at pH 7.5. The enzyme activity was completely lost with *p*-chloromercuribenzoate (1 mM).

Substrate specificity

Table III shows the substrate specificity of the *N*-carbamyl-amino acid amidohydrolase. This *N*-carbamyl-amino acid amidohydrolase was found to be an L-specific enzyme and various *N*-carbamyl-L-α-amino acids were hydrolyzed to the corresponding L-α-amino acids, including both aliphatic and aromatic amino acids, but *N*-carbamyl-β-alanine was not hydrolyzed.

Discussion

The L-specific *N*-carbamyl-α-amino acid amidohydrolase of *Pseudomonas* sp. strain NS671 was purified to homogeneity from cells of *E. coli* in which the gene was expressed.

From measurements of the relative molecular mass of the purified enzyme by gel filtration chromatography and SDS–polyacrylamide gel electrophoresis, the enzyme appeared to be a homodimeric protein with subunit molecular mass of 45 kDa. Recently, Ogawa and Shimizu[2] reported a β-ureidopropionase (*N*-carbamyl-β-alanine amidohydrolase) with *N*-carbamyl-L-α-amino acid amidohydrolase activity from *Pseudomonas putida* IFO12996. The β-ureidopropionase is also a dimer with two identical subunits of a molecular mass of 45 kDa. The *N*-carbamyl-L-α-amino acid amidohydrolase of strain NS671 was different from the β-ureidopropionase of strain IFO12996 because it showed no activity toward *N*-carbamyl-β-alanine.

The *N*-carbamyl-L-amino acid amidohydrolase of strain NS671 required Co^{2+}, Mn^{2+}, or Ni^{2+} for its activity, and the requirement was similar to a thermostable enzyme of *Bacillus stearothermophilus* NS1122A[7] and β-ureidopropionase of *P. putida* IFO12996.[2] Among *N*-carbamyl-L-amino acid amidohydrolases purified partially,[1,9-12] the enzymes from *B. brevis* AJ-12299[9] and *Zymobacterium oroticum*[11] required Mn^{2+} or Fe^{2+} for their activities.

The *N*-carbamyl-amino acid amidohydrolase of strain NS671 had a broad specificity for *N*-carbamyl-L-α-amino acids. In this regard, the enzyme of strain NS671 was different from that of *B. stearothermophilus* NS1122A,[7] which acted mainly on aliphatic *N*-carbamyl-L-amino acids. The enzyme of strain NS671 was also distinct from the ureidosuccinase (*N*-carbamyl-L-aspartate amidohydrolase) of *Z. oroticum*,[11] which showed high substrate specificity.

An SH-reagent, *p*-chloromercuribenzoate, inhibited this reaction by 100% at 1 mM, suggesting that SH-groups of the enzyme are involved in its activity. The peptide estimated from the gene, *hyuC*, for *N*-carbamyl-L-amino acid amidohydrolase contains 5 Cys.

The activity of *N*-carbamyl-L-amino acid amidohydrolase was inhibited by ATP. The dipeptidase from pig kidneys is reported to be competitively inhibited by phosphate and nucleoside phosphate esters such as ATP.[13] The reaction catalyzed by dipeptidase was analogous to the reaction by the *N*-carbamyl-L-amino acid amidohydrolase with respect to hydrolyzing amide bond. The dipeptidase required Zn^{2+} ion to restore full activity, while this *N*-carbamyl-L-amino acid amidohydrolase required Mn^{2+} ion and not Zn^{2+} ion for the activity.

Acknowledgment. The authors thank Dr. Takakazu Kojima of our laboratory for helpful discussions.

References

1) C. Syldatk, A. Läufer, R. Müller, and H. Höke, *Adv. Biochem. Eng./Biotechnol.*, **41**, 29–75 (1990).
2) J. Ogawa and S. Shimizu, *Eur. J. Biochem.*, **223**, 625–630 (1994).
3) T. Ishikawa, K. Watabe, Y. Mukohara, S. Kobayashi, and H. Nakamura, *Biosci. Biotech. Biochem.*, **57**, 982–986 (1993).
4) K. Watabe, T. Ishikawa, Y. Mukohara, and H. Nakamura, *J. Bacteriol.*, **174**, 962–969 (1992).
5) K. Watabe, T. Ishikawa, Y. Mukohara, and H. Nakamura, *J. Bacteriol.*, **174**, 3461–3466 (1992).
6) J. Sambrook, E. F. Fritsch, and T. Maniatis, in "Molecular Cloning: A Laboratory Manual," 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989, p. A.3.
7) T. Ishikawa, Y. Mukohara, K. Watabe, S. Kobayashi, and H. Nakamura, *Biosci. Biotech. Biochem.*, **58**, 265–270 (1994).
8) O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265–275 (1951).
9) A. Yamashiro, K. Kubota, and K. Yokozeki, *Agric. Biol. Chem.*, **52**, 2857–2863 (1988).
10) K. Yokozeki, Y. Hirose, and K. Kubota, *Agric. Biol. Chem.*, **51**, 737–746 (1987).
11) I. Liebermann and A. Kornberg, *J. Biol. Chem.*, **212**, 909–920 (1955).
12) K. Yokozeki and K. Kubota, *Agric. Biol. Chem.*, **51**, 721–728 (1987).
13) C. Harper, A. René, and B. J. Campbell, *Biochim. Biophys. Acta*, **242**, 446–458 (1971).

Downloaded by [North West University] at 16:11 19 December 2014

NII-Electronic Library Service