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R-Stereoselective Amidase from *Rhodococcus erythropolis* No. 7 Acting on 4-Chloro-3-Hydroxybutyramide

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Ethyl (*S*)-4-chloro-3-hydroxybutyrate is an intermediate for the synthesis of Atorvastatin, a chiral drug used for hypercholesterolemia. A *Rhodococcus erythropolis* strain (No. 7) able to convert 4-chloro-3-hydroxybutyronitrile into 4-chloro-3-hydroxybutyric acid has recently been isolated from soil. This activity has been regarded as having been caused by the successive actions of the nitrile hydratase and amidase. In this instance, the corresponding amidase gene was cloned from the *R. erythropolis* strain and expressed in *Escherichia coli* cells. A soluble active form of amidase enzyme was obtained at 18°C. The Ni column-purified recombinant amidase was found to have a specific activity of 3.89 U/mg toward the substrate isobutyramide. The amidase was found to exhibit a higher degree of activity when used with mid-chain substrates than with short-chain ones. Put differently, amongst the various amides tested, isobutyramide and butyramide were found to be hydrolyzed the most rapidly. In addition to amidase activity, the enzyme was found to exhibit acyltransferase activity when hydroxyl amine was present. This dual activity has also been observed in other enzymes belonging to the same amidase group (E.C. 3.5.1.4). Moreover, the purified enzyme was proven to be able to enantioselectively hydrolyze 4-chloro-3-hydroxybutyramide into the corresponding acid. The e.e. value was measured to be 52% when the conversion yield was 57%. Although this e.e. value is low for direct commercial use, molecular evolution could eventually result in this amidase being used as a biocatalyst for the production of ethyl (*S*)-4-chloro-3-hydroxybutyrate.

Keywords: Amidase, 4-chloro-3-hydroxybutyramide, enantioselective, *Rhodococcus erythropolis*

Although nitrile compounds are abundant in nature, the pharmaceutical and chemical industries are also known to produce synthetic nitrile compounds [1]. These nitrile compounds serve as carbon and energy sources for various microorganisms. There are two known nitrile-assimilation pathways [3, 5, 20]: one utilizes nitrilase (E.C. 3.5.5.1) to convert nitriles to corresponding acids, and the other utilizes nitrile hydratase (NHase, E.C. 4.2.1.84) to hydrate nitriles to the corresponding amides, and amidase (E.C. 3.5.1.4) to hydrolyze amides to the corresponding acids.

These nitrile-metabolizing enzymes represent very important biocatalysts in organic chemistry. More to the point, nitrilase has been commercially utilized to produce (*R*)-mandelic acid and (*R*)-3-chloromandelic acid [30]. Meanwhile, NHase has been used in conjunction with the large-scale production of acrylamides and nicotinamides [14, 29]. Moreover, numerous amidases have been exploited in conjunction with the production of optically active compounds from corresponding racemic amides [7, 18, 19, 25, 26]. Amidase enzymes have in particular been singled out for their high enantioselectivity. Enantioselective amidases have been reported in some microorganisms, including *Brevibacterium* sp. R321, *Pseudomonas chlororaphis* B23, and *Rhodococcus rhodochrous* J1.

Many microbial amidases have been purified and characterized. To this end, these can be divided into two types based on their catalytic activity functions [9]. The first type includes aliphatic amidases that only hydrolyze short-chain aliphatic amides; the second one includes aliphatic amidases that hydrolyze mid-chain amides and arylamides, and which are coupled with the nitrile hydratases involved in nitrile metabolism.

Ethyl (*S*)-4-chloro-3-hydroxybutyrate [(*S*)-ECHB] is a chiral intermediate used in the production of Atorvastatin, a chiral drug for hypercholesterolemia [23]. (*S*)-ECHB can be synthesized through two successive enzyme reactions and a chemical reaction (Scheme 1). The efficient production of (*S*)-ECHB requires that enantioselective amidase be used to convert racemic 4-chloro-3-hydroxybutyramide (CHBamide) into (*S*)-4-chloro-3-hydroxybutyric acid (CHBacid).

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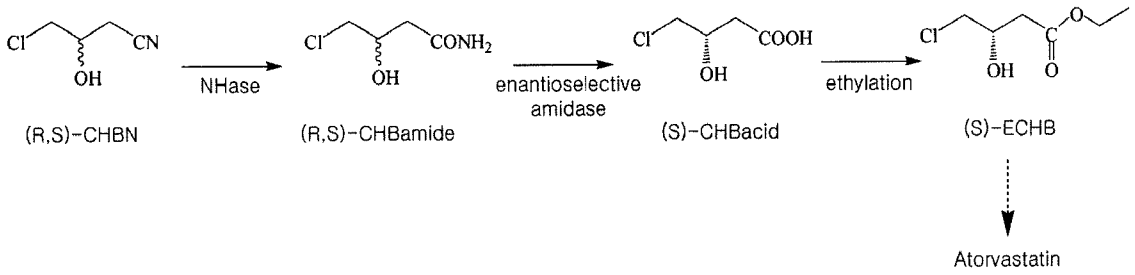


Scheme 1. Conversion of CHBN to (*S*)-ECHB.
(*S*)-CHBacid can be synthesized from racemic CHBN with NHase and enantioselective amidase. (*S*)-ECHB can be made from (*S*)-CHBacid by ethylation reaction and be used for the synthesis of Atorvastatin.

This study reveals that, through the catalytic activity of the NHase and amidase, *Rhodococcus erythropolis* No. 7 can convert 4-chloro-3-hydroxybutyronitrile (CHBN) into CHBacid [23]. In this research, the relevant amidase enzyme was expressed in *E. coli* and characterized biochemically, before being applied to the enantioselective conversion of CHBamide to CHBacid.

Materials and Methods

Chemicals

CHBN, CHBamide, and CHBacid were supplied by Equispharm, Inc. (Korea). All other organic compounds were obtained from commercial sources and were of reagent grade.

Bacterial Strain, Plasmid, and Culture Conditions

E. coli BL21 (DE3) was used as a host for the recombinant plasmid. The plasmid pET22 was used as an expression vector. The recombinant *E. coli* BL21 (DE3) was cultured at 18°C on a Luria-Bertani medium (10 g tryptone, 5 g yeast extract, 5 g NaCl per liter) containing 100 μg/ml of ampicillin.

Construction of Expression Plasmid

The amidase gene of the *R. erythropolis* strain (No. 7) was amplified using the PCR primers amiF (5'-GATCCATATGGCGAC-AATCCGACCT-3') and amiR (5'-GATGAATTCAAGGCGGGGCT-GAGTTG-3'). PCR was performed with TaKaRa *Taq* (Takara Bio Inc., Shiga, Japan) to conduct 30 cycles of 95°C for 60 sec, 43°C for 60 sec, and 72°C for 90 sec. The amplified PCR product was then digested with NdeI and EcoRI, and then purified with an AxyPrep DNA gel extraction kit (Axygen Scientific, Inc., Union City, CA, U.S.A.). The amplified DNA was inserted downstream of the T7 promoter in the pET22 [16, 21, 24].

Expression of Recombinant Amidase in *E. coli*

E. coli BL21 (DE3)/pET22-ami was cultured at either 18°C or 37°C in LB broth supplemented with 100 μg/ml ampicillin. The cultures were grown until the OD 600 nm reached a value of 0.5 with shaking (230 rpm); thereafter, IPTG was added to a final concentration of 1 mM [2]. Induction was then carried out for 5 h and 20 h at 37°C and 18°C, respectively. One hundred ml of each culture was then centrifuged (10 min, 6,000 ×*g*) and resuspended in 4 ml of a potassium phosphate buffer (10 mM, pH 7.5). The cells were sonicated and a soluble fraction was obtained through centrifugation (15 min, 10,000 ×*g*).

Purification of Recombinant Amidase

The amidase protein found in the soluble fraction was purified as follows. First, a protein mixture was loaded onto a 5-ml Ni-NTA column (QIAGEN Inc., Valencia, CA, U.S.A.) equilibrated with a 50 mM imidazole buffer (pH 8.0) containing 50 mM NaH_2PO_4 and 300 mM NaCl. The recombinant amidase No. 7 was eluted from the column by applying a 200 mM imidazole buffer. The active fractions were then pooled and desalted.

The protein concentration was determined using a Bradford protein assay kit (Bio-Rad Laboratories, Hercules, CA, U.S.A.) in which bovine serum albumin served as the standard protein.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed on polyacrylamide slab gels that used a Tris-glycine buffer system. The protein samples (25 μg) were then loaded on a separation gel (12%).

Amidase Activity Assay

The assay of hydrolytic activity was performed by detecting ammonia using the method developed by Fawcett and Scott [8]. The purified enzyme was added to 1 ml of phosphate buffer (10 mM, pH 7.5) containing substrates (5 mM concentration), and the reaction mixtures were incubated with rotation at 40°C. At various time intervals, 100-μl portions of the reaction mixture were extracted and mixed together with 0.5 ml of solution A (10 g of phenol, 0.4 g of sodium nitroprusside per liter) and 0.5 ml of solution B (5 g of NaOH, 7 ml of sodium hypochlorite solution per liter). After 30 min incubation at room temperature, the absorbance of the sample was determined at 625 nm, and was then compared with a standard curve for NH_4OH (0–0.5 mM). One unit of enzyme activity was defined as the amount of enzyme needed to produce 1 μmole of NH_3 per min.

The assay of the acyl transfer activity was determined spectrophotometrically using the method of Fournand *et al.* [11]. One ml of a potassium phosphate buffer (10 mM, pH 7.5) containing 10 mM substrate was mixed with 1 ml of a hydroxylamine hydrochloride (1 M) solution adjusted to pH 7.0 with 10 N NaOH. The enzyme was added and incubated at 40°C. At regular intervals, 500 μl portions of the reaction mixture were extracted and added to 1 ml of the acidic $FeCl_3$ solution (355 mM $FeCl_3$ in 0.65 M HCl). Thereafter, the absorbance of the sample was measured at 500 nm, which was then compared with the standard curves for hydroxamate (0–1 mM). One unit of enzyme activity was defined as the amount of enzyme needed to produce 1 μmole of monohydroxamate-iron (III) per min.

Determination of Substrate Specificity

Short-chain amides (acetamide, acrylamide, and propionamide), mid-chain amides (butyramide and isobutyramide), and an aromatic amide (benzamide) were used as substrates. The amount of ammonia or monohydroxamate-iron (III) complex utilized was determined using the above-mentioned method.

Effects of Temperature and pH on Purified Amidase

In order to determine the optimal temperature for amidase activity, 1 ml portions of the reaction mixture containing 5 mM isobutyramide and purified enzyme (1.05 μg) in a 10 mM potassium phosphate buffer, pH 7.5, were incubated for 30 min at 10°C–70°C.

The following buffer solutions were used to determine the optimum pH for amidase activity: 50 mM of a sodium acetate buffer at pH 4–6; 50 mM of a KH_2PO_4-K_2HPO_4 buffer at pH 6–9; 50 mM of a Tris-HCl buffer at pH 8–9.5; 50 mM K_2HPO_4-K_3PO_4 buffer at pH 9.5–11. Each 1 ml of the reaction mixture containing 5 mM isobutyramide and purified enzyme (1.05 μg) was then incubated for 30 min at 40°C.

In order to determine temperature stability, 990 μl portions of a potassium phosphate buffer (10 mM, pH 7.5) containing purified enzyme (1.05 μg) were incubated for 30 min at temperatures varying between 10°C and 60°C. The pretreated enzyme was reacted with 10 μl of isobutyramide (500 mM) for 30 min at 40°C.

The following buffer solutions were used for the determination of the pH stability for amidase activity: 50 mM of a KH_2PO_4-K_2HPO_4 buffer at pH 6–8; and 50 mM of a K_2HPO_4-K_3PO_4 buffer at pH 9–12. Each 100 μl pH buffers containing purified enzyme (10.5 μg) were then incubated for 30 min on ice. Thereafter, 10 μl portions of pretreated enzyme were reacted with 10 μl of isobutyramide (500 mM) and 980 μl of a potassium phosphate buffer (10 mM, pH 7.5) for 30 min at 40°C.

Effects of Potential Inhibitors on Purified Amidase

To determine the effects of possible inhibitors on the amidase, 2.1 μg of the purified enzyme in a 10 mM potassium phosphate buffer, pH 7.5, was mixed with EDTA, 1,10-phenanthroline, iodoacetamide, PMSF, or mercury chloride in a total volume of 990 μl and incubated for 30 min at 4°C. After subsequent addition of 10 μl of 500 mM isobutyramide, the residual amidase activity was measured for 30 min at 40°C.

Determination of Kinetic Parameters

The K_{cat} and K_m values for the various substrates of the purified amidase were determined. The final concentrations were found to be acetamide 0.5–7 mM; acrylamide 0.1–0.5 mM; benzamide 0.02–0.25 mM; propionamide 0.02–0.2 mM; butyramide 0.02–0.25 mM; and isobutyramide 0.02–0.25 mM. Purified enzyme was then added and reacted for 10 min at 40°C. The K_{cat} and K_m values of the various substrates of the enzyme were determined using a Lineweaver-Burk plot of the experimental values.

Hydrolysis of 4-Chloro-3-Hydroxybutyramide

The hydrolytic activity toward CHBamide was determined using thin layer chromatography (TLC). One ml of the reaction mixture containing 157.5 μg of purified enzyme, 100 μl of CHBamide, and 750 μl of potassium phosphate buffer (10 mM, pH 7.5) was incubated at 40°C. At various intervals, 30-μl samples were taken and added to a silica gel plate (Merck KGaA, Darmstadt, Germany). It was developed with hexane/ethyl acetate/acetic acid (2:3:0.5) and the spots were detected using *p*-anisaldehyde/acetic acid/95% ethanol/sulfuric acid (9.2:3.75:338:12.5).

Enantioselective Biotransformation of CHBamide

Enantioselectivity was determined by assaying the amount of ammonia released from (*R*)- or (*S*)-CHBamide. One ml of a reaction mixture containing purified enzyme (157.5 μg), each form of CHBamide (2.3 mg), and a potassium phosphate buffer (10 mM, pH 7.5) was incubated at 40°C. At various intervals, samples of 100 μl were analyzed for ammonia.

Results and Discussion

Production of Amidase No. 7 in *E. coli*

As previously reported, *R. erythropolis* No. 7 was found to exhibit both NHase and amidase activities [23]. The

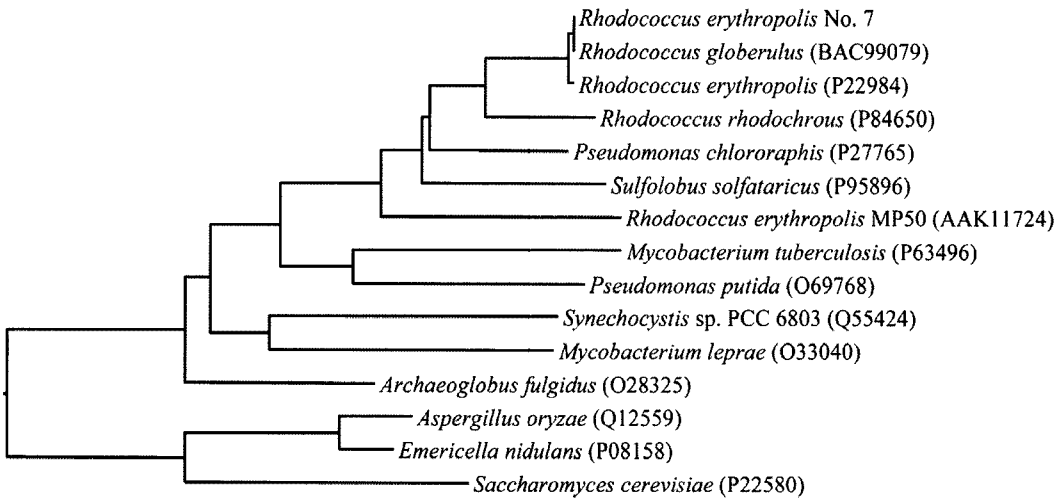


Fig. 1. Phylogenetic tree of *R. erythropolis* No. 7 amidase and related amidases.
Multiple sequence alignment was done for amidase No. 7 and 14 amidases using the Clustal method stipulated in the DNASTAR program.

corresponding amidase gene was cloned, and found to consist of 1,566-bp DNA encoding protein with 521 amino acids. The nucleotide sequence was submitted to GenBank under the accession number EU029986. To date, more than 26 enzymes belonging to the amidase (E.C. 3.5.1.4) group have been found and characterized. The molecular size of these enzymes has been proven to be very diverse (38–61 kDa), and as such can be divided into two subgroups; namely, small amidases (~38 kDa) and large amidases (~55 kDa). Amidase No. 7 belongs to the large amidase subgroup, and a multiple sequence alignment revealed that it shared the greatest number of similarities with the *Rhodococcus globerulus* amidase (BAC99079) and *Rhodococcus erythropolis* amidase (P22984), which featured identities of 99.4% and 96.9%, respectively (Fig. 1). Although *R. globerulus* amidase has been reported, its detailed biochemical properties have yet to be elucidated [28]. Meanwhile, as many as three *R. erythropolis* amidases have been reported. As one (Q01360) of these enzymes belongs to the small amidase subgroup and features a very different protein sequence from the other two, it has been omitted from the sequence alignment found in Fig. 1. The second one, amidase MP50, was found to be similar in protein size and sequence identity (31.1%) to amidase No. 7 [12, 27]. The last strain (P22984) was uncovered to be the one most similar (96.9% identity) to amidase No. 7.

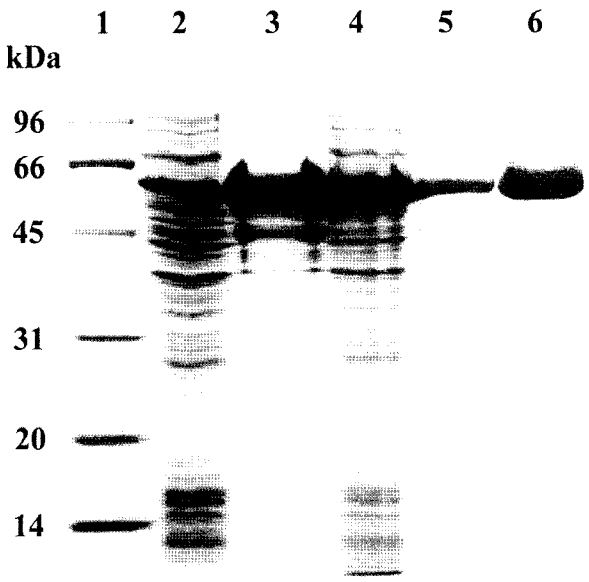
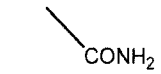
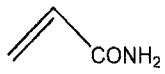
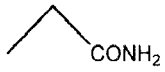
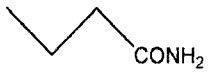
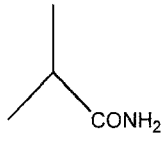
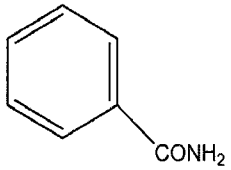


Fig. 2. SDS-PAGE of recombinant amidase No. 7.
Lane 1, size standard; lanes 2 and 3, soluble fraction and insoluble fraction from culture induced with 1 mM IPTG for 5 h at 37°C, respectively; lanes 4 and 5, soluble fraction and insoluble fraction from culture induced with IPTG for 20 h at 18°C, respectively; lane 6, purified enzyme from Ni-NTA column.

It has been characterized biochemically and utilized industrially [11, 15].

Table 1. Substrate specificity of purified amidase No. 7.

Substrate	Structural formula	Hydrolytic activity[a] (U/mg)	Molar absorptivity of hydroxamate/iron (III) complex (10^2 $M^{-1}cm^{-1}$)	Acyl transfer activity[c] (U/mg)
Acetamide		0.21	3.22	0.17
Acrylamide		0.39	11.22[b]	0.10
Propionamide		1.56	10.29[b]	1.66
Butyramide		1.58	n.d.	n.d.
Isobutyramide		3.89	n.d.	n.d.
Benzamide		0.79	3.78	0.53

[a]Hydrolytic activities were calculated from released ammonia determination.
[b]ε_M values of iron (III)/acrylohydroxamate complex and iron (III)/propionohydroxamate complex were referred from the literature [11].
[c]Acyl transfer activities were calculated with each ε_M.
n.d., not determined.

The amidase gene was then subcloned in a pET22 vector and expressed in *E. coli* BL21 (DE3). When the recombinant *E. coli* was cultured and induced using IPTG at 37°C, most of the amidase protein emerged as an insoluble inclusion body (Fig. 2, lane 3). Conversely, most of the amidase protein emerged as a soluble active enzyme when the cell was cultured and induced at 18°C (Fig. 2, lane 4). The lowering of the induction temperature to 18°C slowed the *E. coli* cell's production of the amidase protein, thus favoring its proper folding into an active form rather than its aggregation to an inclusion body. Amidase activity in the soluble fraction was measured to be as much as 0.97 U/mg protein toward isobutyramide. As the recombinant amidase had His-tag at its C-terminal end, it could be efficiently purified using Ni-NTA column chromatography (Fig. 2, lane 6). The purified amidase was uncovered to have a specific activity of 3.89 U/mg toward isobutyramide. This is comparable to the *R. erythropolis* MP50 amidase (4.14 U/mg, phenylacetamide), *Sulfolobus tokodaii* amidase (14.8 U/mg, benzamide), and the *Microbacterium* sp. AJ115 amidase (9.01 U/mg, acetamide).

Substrate Specificity of Amidase No. 7

Hydrolytic activities toward various amides were measured in order to ascertain the substrate specificity of purified amidase No. 7 (Table 1). Although purified amidase No. 7 was found to hydrolyze isobutyramide, butyramide, and propionamide rapidly, it did so in a much slower fashion in the case of acetamide, acrylamide, and benzamide. These results prove that amidase No. 7 belongs to the second aliphatic amidase group of hydrolyzing mid-chain amides [9].

Some *Rhodococcus* amidases are known to exhibit acyl transfer activity in the presence of hydroxylamine [10, 13]. Purified amidase No. 7 was also found to be able to catalyze an acyl transfer reaction between some amides and hydroxylamine (Table 1). The fact that this amidase enzyme exhibited both amidase and acyl transfer activities is indeed an interesting development. However, these two activities were, in fact, with the notable exception of the last step, mediated using the same mechanism. Whereas the former used H_2O molecules during the deacylation step, the latter used hydroxyl amine, thus resulting in the acyl transfer reaction. To this end, this acyl transfer activity can serve as a tool with which to screen an active and enantioselective amidase enzyme [31].

Effects of Temperature and pH

As isobutyramide is widely regarded to be the best substrate (Table 1), it was thus used in the following experiments. The hydrolytic activity of the enzyme continuously increased up to temperatures of 55°C, but it subsequently decreased rapidly at temperatures in excess of 60°C (Fig. 3A). The optimum pH of the enzyme was

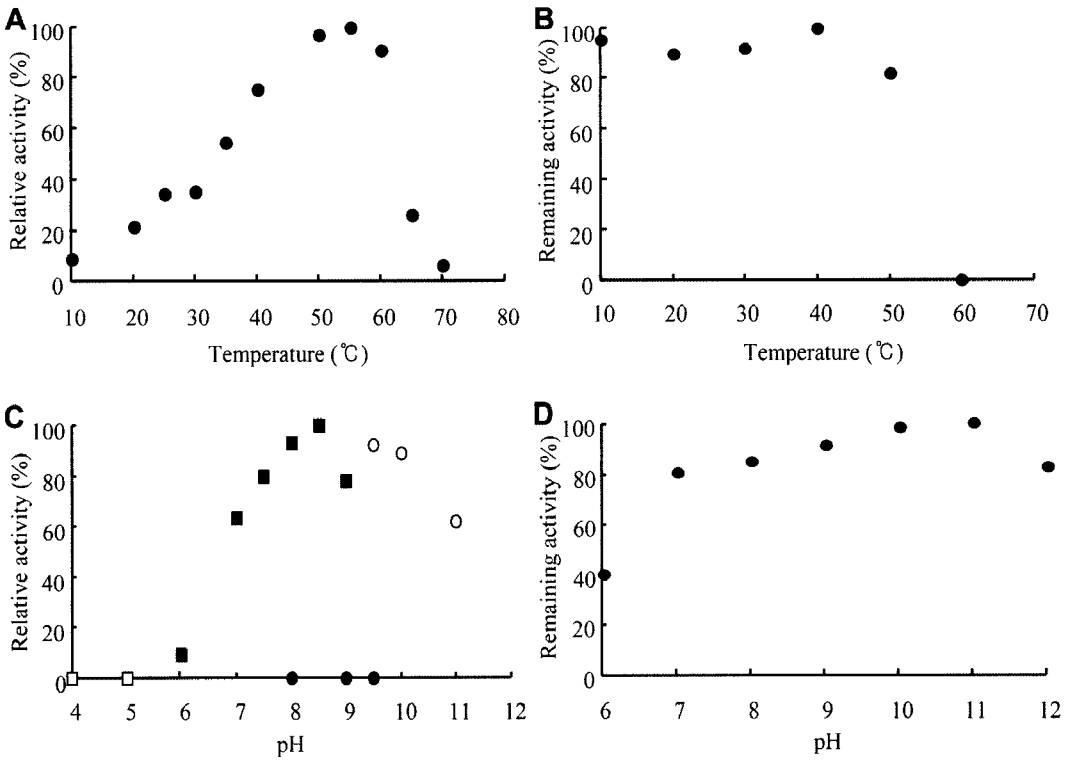


Fig. 3. Effects of temperature and pH on purified amidase No. 7.

A. Effects of temperature on activity; **B** effects of temperature on stability; **C** effect of pH on activity using 50 mM sodium acetate buffer (□), potassium phosphate buffer (■), Tris-HCl buffer (●), and K_2HPO_4-K_3PO_4 buffer (○); **D** effects of pH on stability.

determined to be pH 8.5 (Fig. 3C). No hydrolytic activity whatsoever was uncovered when the enzyme reaction was performed in a Tris-HCl buffer. This result has been linked to the possibility that, given its structural similarity with the isobutyramide substrate, the Tris molecule itself may act as a competitive inhibitor. In this regard, a potassium phosphate buffer was used in lieu of a Tris-HCl one in this particular study. To this end, the purified enzyme was found to be stable at temperatures of up to 50°C (Fig. 3B) and at pH 7–12 for 30 min (Fig. 3D).

Effects of Chemical Reagents

Various chemical reagents were added to the amidase enzyme in order to explain the reaction mechanism of the enzyme (Fig. 4). Of these, mercury chloride was found to completely inhibit amidase activity, and iodoacetamide did so in a partial manner. The PMSF and metal chelator were revealed to have little effect on enzyme activity. These results prove that this amidase enzyme is a typical sulfhydryl enzyme, and that the cysteine residue might represent an important amino acid in the active site.

As mentioned above, the amidases were divided into two groups [9]. The first group of amidases featured an amidase signature sequence of a Ser-cisSer-Lys catalytic triad and/or Cys-cisSer-Lys center [6]. Amidase No. 7 was found to belong to the second amidase group, which contains most of the amidases related to nitrile metabolism. This group of amidases has a catalytic site formed by a Glu-Cys-Lys triad [4, 17, 22]. The sequence alignment showed that Glu191, Cys203, and Lys96 consisted of an active center (Fig. 5).

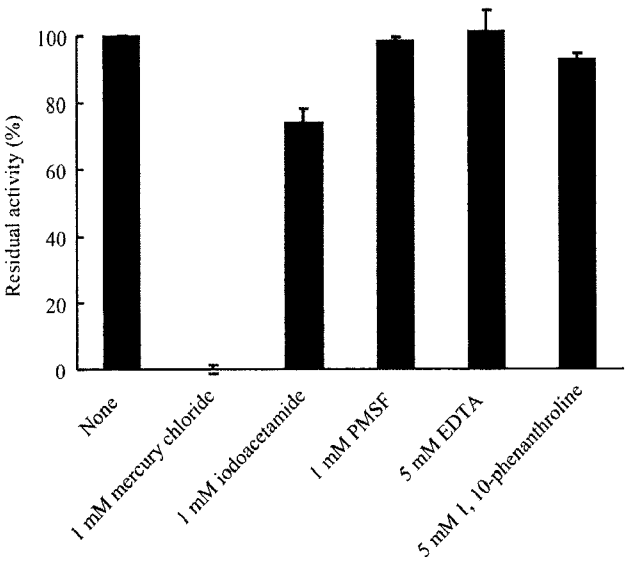


Fig. 4. Effects of chemical reagents on purified amidase No. 7.
Residual activities were calculated relative to the activity of the untreated enzyme (100% corresponds to 3.89 U/mg toward isobutyramide substrate).

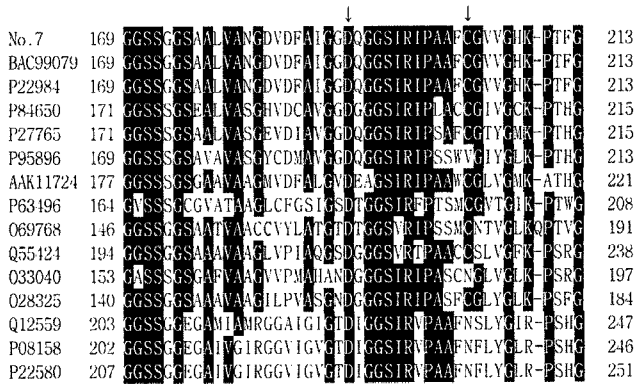


Fig. 5. Signature sequence of amidase No. 7.
Signature sequences of amidase No. 7 and 14 other amidases were compared. Amino acids that conserved more than 10 amidases were shadowed.

Kinetic Study of Amidase No. 7

The turnover number (K_{cat}) and Michaelis constant (K_m) of the enzyme toward various substrates were also calculated. As expected, the K_{cat}/K_m value for isobutyramide was the highest (31.56 $sec^{-1}mM^{-1}$). The amidase enzyme hydrolyzed isobutyramide, butyramide, and propionamide molecules at a relatively high turnover rate. Although the turnover rate for benzamide was very low, the binding affinity was very high (K_m=0.04 mM). Moreover, its K_{cat}/K_m value was comparable to that of butyramide. These kinetic data appear to support the assumption that this enzyme belongs to the second group of aliphatic amidases.

Hydrolysis of CHBamide

The purified amidase No. 7 was found to be able to convert racemic CHBamide into CHBacid (Fig. 6A). The amidase was unable to convert CHBamide when the amidase inhibitor $HgCl_2$ was added to the reaction mixture at 0 min, thus clearly proving that the hydrolysis reaction was carried out by the purified amidase No. 7.

The enzyme hydrolyzed both (*R*)- and (*S*)-CHBamides when these were used as substrates (Fig. 6B). However, it hydrolyzed (*R*)-CHBamide more rapidly than the (*S*)-compound. The e.e. value was uncovered to be 52% when the conversion yield reached 57%. These results clearly prove that amidase No. 7 exhibits enantioselectivity toward (*R*)-CHBamide.

Table 2. Kinetic parameters of purified amidase No. 7 toward various substrates.

Substrate	K_{cat} (sec^{-1})	K_m (mM)	K_{cat}/K_m ($sec^{-1}mM^{-1}$)
Acetamide	0.21	5.57	0.04
Acrylamide	0.58	4.79	0.12
Propionamide	4.03	1.30	3.10
Butyramide	2.04	0.18	11.33
Isobutyramide	5.68	0.18	31.56
Benzamide	0.48	0.04	12.00

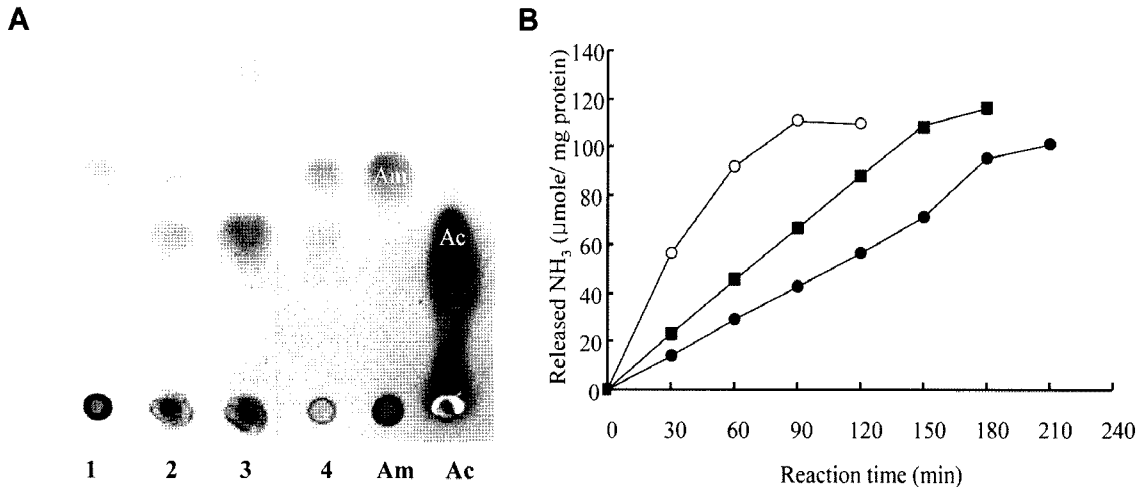


Fig. 6. Hydrolysis of CHBamide.
A. TLC analysis of the hydrolysis of CHBamide. Lanes 1, 2, and 3 were 0 min, 60 min, and 120 min reaction mixtures, respectively; lane 4 was a sample of 120 min reaction with 1 mM $HgCl_2$; Am and Ac are CHBamide and CHBacid, respectively. **B**. The purified enzyme's rates of degradation of racemic CHBamide (■), (*R*)-CHBamide (○), and (*S*)-CHBamide (●) were ascertained through released NH_3 determination.

The enantioselectivity and catalytic activity of the enzyme could be increased by optimizing the condition of the enzyme reaction and/or performing a molecular evolution of the enzyme itself, and it was found to be used in the production of the important chiral intermediate (*S*)-ECHB.

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