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Discovery of a novel *N*-iminylamidase activity: substrate specificity, chemicoselectivity and catalytic mechanism

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Abstract

Enzymatic hydrolysis of the *N*-iminylamide was investigated in this study. An enzyme possessing *N*-iminylamidase activity from pig liver was purified to electrophoretic homogeneity. This enzyme was also active, however, with imides and appears to be identical to pig liver imidase. The identification was confirmed by copurification of enzyme activities and by specificities of typical substrates of mammalian imidase, such as phthalimide, dihydrouracil, and maleimide. The hydrolysis of 3-iminoisoindolinone was further analyzed by HPLC, ^{13}C NMR spectrometry, and LC–MS measurements to determine its chemicoselectivity. All data indicated that this enzyme chemicoselectively catalyzed the hydrolysis of the *N*-iminylamide to produce the compound bearing the diamine and carboxylate group. The pH profiles of this enzyme suggest that one of the protons of 3-iminoisoindolinone was important to promote the ring-opening process of this substrate. These results constituted a first study on the enzymatic hydrolysis of compounds bearing the *N*-iminylamide functional group.

Keywords: *N*-iminylamidase; Imidase; Hydantoinase; Dihydropyrimidinase

New enzymatic activities likely evolve by the recruitment of a protein catalyzing the same type of chemical reaction but possessing different substrate specificity [1,2]. This hypothesis has been supported with increasing databases of the protein sequence and structure, including the enolase, amidohydrolase, thiyl radical crotonase, vicinal-oxygen-chelate, and Fe-dependent oxidase superfamilies [3]. The diversity of enzyme catalytic function is remarkable, particularly when one considers that ancestral life forms must have started with a much smaller ensemble of proteins. A little change of structure or sequence of protein is a driving force that might lead proteins to be with different substrate specificity or form a novel enzyme in evolution [3]. Thus, in search of the existence of new enzymatic activity or a new enzyme is a direct way to increase the knowledge of the structure–function relationship of these proteins.

One of frequent occurrence in structurally characterized enzymes, amidohydrolase superfamily, has recently emerged as a model for a wide range of studies in evolution of TIM-barrel enzymes [4] and sequence/structure relationships [5]. These detailed analyzes have summarized that they catalyze a similar biochemical reaction in acting on the amide bond but without overlapping substrates in spite of they possessing highly conserved sequence and structure identity. More studies are needed to understand the reactions in amidohydrolase superfamily.

In recent years, several amidohydrolases and amidases were found from various species. For examples, barbiturase [6] and ureidomalonase [7] from *Rhodococcus erythropolis* JCM 3132; half-amidase [8] and imidase [9] from *Blastobacter* sp. strain A17p-4; and phenylhydantoinase [10] from *Escherichia coli* have been discovered. These bacterial enzymes were shown to catalyze the

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compounds bearing amide functional group with a different type of carbon skeleton. In accord with our interest in exploring a new enzyme and a new enzymatic reaction, we sought to obtain a novel *N*-iminylamidase and to use it for an investigation of the reaction of enzyme-catalyzed hydrolysis of 3-iminoisoindolinone bearing *N*-iminylamide functional group from pig liver. Since then, no follow-up article has appeared on the cyclic *N*-iminylamide-degrading activity.

Of specific interest is the finding an enzyme with novel activity in mammal, especially is capable of serving in a role for detoxication of xenobiotics [11]. Here, we reported an enzyme, likely to be identical to pig liver imidase, which was able to catalyze hydrolysis of 3-iminoisoindolinone, a compound without imide functional group. Thus, we put forward an extended substrate range of mammalian imidases from the linear [11], the heterocyclic imide [11], maleimide [12], organic cyclic carbonates [13] to *N*-iminylamide. In this paper, we initiated the first study on *N*-iminylamide hydrolytic activity. Furthermore, a detailed regiochemical reaction of this enzyme was sketched by several methods. These results might also provide a novel biosynthetic method to obtain a compound with diamine and carboxylate group.

Materials and methods

Materials

3-Iminoisoindolinone (97%) was a product of Aldrich Chemical (USA). Candidate substrates for the enzyme, such as barbituric acid, allantoin, dihydroorotate, uracil, pyromellitic diimide, 5-bromouracil, 2,3-naphthalene-dicarboximide, 1,8-naphthalimide, 3,4,5,6-tetrachlorophthalimide, 4-amino-1,8-naphthalimide, 2*H*-1,3-benzoxazine-2,4(3*H*)dione, biuret, bemegride, 5,5-dimethyl-hydantoin, uridine, 5-ethyl-methyl-hydantoin, parabanic acid, rhodanine, 2,4-thiazolidinedione, *N*-methylmaleimide, *N*-cyclohexylmaleimide, oxindole, and phthalic anhydride, were obtained from Sigma (USA) or Aldrich. Octyl-Sepharose CL-4B, DEAE–Sephacel, chelating-Sephacel (fast flow), and HiTrap desalting column were purchased from Pharmacia. Bis-Tris propane, sodium acetate, PMSF, and EDTA were purchased from Sigma. Sodium chloride, ammonia sulfate, sodium hydroxide, potassium phosphate, glycine, sodium dodecyl sulfate (SDS),[1] and zinc acetate were obtained from J.T. Baker (USA). All other reagents were of the highest grades commercially available.

[1] *Abbreviations used:* Tris–HCl, Tris(hydroxymethyl)aminomethane hydrochloride; DEAE, diethylamino-ethyl; SDS, sodium dodecyl sulfate; Bis-Tris propane, 1,3-bis(tris(hydroxymethyl) methylamino) propane; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; EDTA, (ethylenedinitrilo) tetraacetic acid.

Enzyme assay

A rapid spectrophotometric assay was devised in which the decrease in absorbance at 310 nm was measured upon hydrolysis of 3-iminoisoindolinone as a substrate (Fig. 1). A 2 mM solution of the sparingly soluble 3-iminoisoindolinone was prepared in water and remained stable at 25 °C for several days. To start the reaction, 0.5 ml of this solution was added to 0.5 ml of 0.2 M Bis-Tris propane at pH 8.0 that contained an appropriate amount of enzyme. The reaction was monitored with a Hitachi U 3300 spectrophotometer in a chamber maintained at 25 °C. Under these conditions, a change in A_{310} of 1.92 represents the hydrolysis of 1 μmol of the substrate; initial rates of change were a function of enzyme concentration over the absorbance range of 0.01–0.20 min^{-1} at 310 nm. A unit of activity is defined as the amount of enzyme catalyzing the hydrolysis of 1 μmol of 3-iminoisoindolinone/min. Specific activity is in terms of units of activity/mg of enzyme. The hydrolysis of a number of candidate substrates was assayed spectrophotometrically in an entirely similar manner except for the wavelength used; necessary modifications of the assay are presented as needed for specific compounds.

The pH profile of N-iminylamidase

The initial velocities of *N*-iminylamidase were determined over the pH range of 5.0–10.0. However, the activity of *N*-iminylamidase at pH 5.0–5.5 was too small to detect when 3-iminoisoindolinone was used a substrate. The reaction was buffered with 0.1 M sodium acetate ($pK_a = 4.8$) and 0.1 M Bis-Tris propane (pK_as = 6.8 and 9.0) in desired pH, and appropriate amount of *N*-iminy-

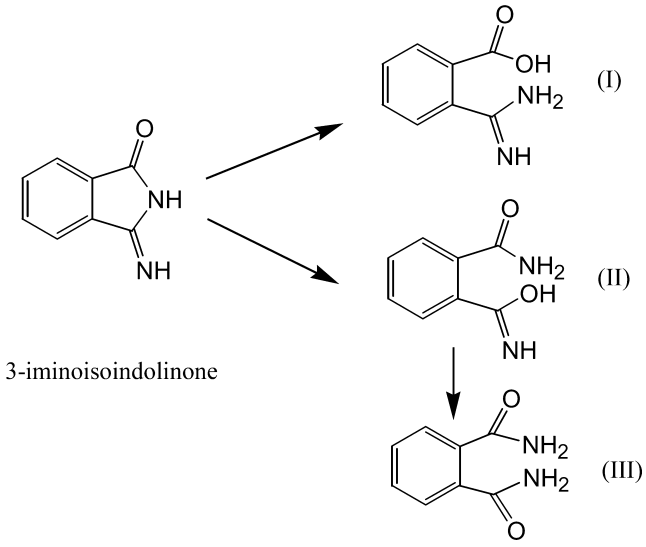

Fig. 1. The possible pathways for the hydrolysis of 3-iminoisoindolinone.

lamidase (about 5–400 μg) was added to start the reaction. The pH of buffers was determined at 25 °C. K_m and V_{max} were obtained by nonlinear regression (enzyme kinetics module of Sigma-Plot) using 10–15 measurements determined at different substrate concentrations. Reaction velocities obtained at each pH value were fitted to the following equation using Sigma-Plot, $(V_{max})_H = V_{max}K_a/K_a+[H^+]$, then p$K_a$ value was given, where $(V_{max})_H$ is the maximal degradative reaction rate at a particular pH, V_{max} is the maximal rate when all the *N*-iminylamidase sites are in the appropriate ionic form, and K_a is the acid dissociation constant for a catalytic residue at the active site [14]. A plot of V_{max}/K_m or K_m as a function of pH was also fitted to this equation in a similar manner.

Analysis of the hydrolytic product of 3-iminoisoindolinone by electrospray ionization mass spectrometry (ESI-MS)

ESI-MS investigations were carried out by means of a quadrupole time-of-flight (Q-TOF) mass spectrometer (Micromass, UK) equipped with an electrospray ion source. Electrospray ionization-mass spectrometry was performed in the positive-ion modes. Sample volumes of 20 μL/min were applied by loop injection. The liquid eluent was water:acetonitrile:1% formic acid (8:1:1, v/v). The collision energy of MS-MS spectra was 10 V and nitrogen was used as nebulizing and drying gas.

Enzyme purification

Fresh pig livers were frozen at −20 °C immediately after being removed from the animals and then stored at −80 °C. All procedures for protein purification were conducted at 4 °C or in an ice bath. The pH of buffers for enzyme purification refers to measurements taken at room temperature. An FPLC system (Pharmacia) was used for column chromatography.

Step 1. *Extract*. *N*-Iminylamidase was extracted from frozen pig liver (about 500 g) with 500 ml buffer A (containing 50 mM phosphate buffer, 1 mM PMSF, and 2 mM EDTA at pH 8). Following mixing with a blender, the suspension was centrifuged at 20,000*g* for 1 h to remove precipitate.

Step 2. *Heat treatment*. The extract was collected and incubated in water bath at 55 °C for 10 min. The suspension was centrifuged at 10,000*g* for 10 min to remove the precipitate.

Step 3. *Salting out*. Ammonium sulfate (equivalent to 35% of saturation) was slowly added to the enzyme extract and stirred for 60 min. The suspension was centrifuged at 20,000*g* for 30 min to remove the precipitate and an additional ammonium sulfate was added into a supernatant fluid to 60% saturation. After gentle stirring for 60 min, the resultant precipitate was collected by centrifugation (20,000*g*, 30 min). The precipitate was dissolved in phosphate buffer (10 mM, pH 7) containing 1.5 M ammonium sulfate, 1 mM PMSF, and 2 mM EDTA (buffer B), and stirred gently for 120 min. Insoluble substances were removed by centrifugation (20,000*g*, 30 min). The dissolution of enzyme precipitate was repeated for four times to ensure that all the *N*-iminylamidase activity was collected in the solution.

Step 4. *Octyl-Sepharose*. The enzyme solution was applied to a column (4.4 × 10 cm) of octyl-Sepharose CL-4B that had been equilibrated with buffer B followed by washing with 150 ml of the same buffer. Protein was eluted with a reverse linear salt gradient of 1.5–0 M ammonium sulfate using 1000 ml each of buffer B and buffer B minus ammonium sulfate. Active fractions (approximately 9 ml each) were pooled (fractions 30–45). The active fractions were desalted by the dialysis (for 4 h; two changes of fresh buffer C containing phosphate buffer 10 mM and 1 mM PMSF, pH 8, 4 L) and the buffer solution was exchanged with buffer C.

Step 5. *DEAE–Sephacel*. The desalted enzyme solution was loaded into a column of DEAE–Sephacel (2.6 × 15 cm, previously equilibrated with buffer C). Protein was eluted with a linear salt gradient of 0–0.3 M sodium chloride using 1000 ml each of buffer C and buffer C plus sodium chloride. The pooled active fractions were applied to next step directly.

Step 6. *Chelating-Sephacel*. The enzyme solution in buffer C was applied to a column (1.6 × 10 cm) of chelating-Sephacel (fast flow) that was treated with one gel volume of 0.2 M zinc acetate and then equilibrated with buffer D (containing phosphate buffer 20 mM, 1 mM PMSF, and 0.5 M NaCl, pH 7). The loaded column was washed with 70 ml buffer D, and the enzyme was eluted with a linear glycine gradient from 0 to 1 M with buffer D and buffer D plus 1 M glycine (total volume 1200 ml). Active fractions were pooled, concentrated by ultrafiltration (YM 100 membrane), and frozen at −80 °C for later analysis. By the standard assay, the specific activity of pig liver *N*-iminylamidase was 2.2 μmol/min/mg in a typical experiment. The results of purification are summarized as Table 1.

Protein purity and molecular weight determination

The purity and subunit molecular weight of *N*-iminylamidase was determined by SDS–PAGE. Gel electrophoresis was performed in a Mini-Protein II Electrophoresis Cell (Bio-Rad, USA). A 10 or 12% acrylamide gel with 4% stacking gel was used with SDS following the method of Laemmli [15]. Protein was stained with Coomassie blue R-250 (National Diagnostics). According to the criteria of SDS–PAGE (Fig. 2, lane 1), *N*-iminylamidase was purified to homogeneity, and the molecular weight for a single polypeptide chain was estimated to be around 56,000.

Determination of native molecular weight of *N*-iminylamidase estimated by use of gel filtration with Sephacryl S-300 (Pharmacia) was about 250,000 (data not

Table 1
Summary of purification of *N*-iminylamidase from pig liver

Step	Total activity (μmol/min)	Total protein (A_{280})	Specific activity (μmol/min/mg)	Yield (%)	Fold of purification
Extract	1300	238500	0.005	100	1.0
Heat treatment	1100	60500	0.018	85	3.6
35% salt out	900	45600	0.020	69	4.0
60% salt out	630	17400	0.036	49	7.2
Octyl-Sepharose	330	1860	0.177	25	35
DEAE–Sephacel	220	590	0.373	17	75
Chelating-Sephacel	145	65	2.23	11	450

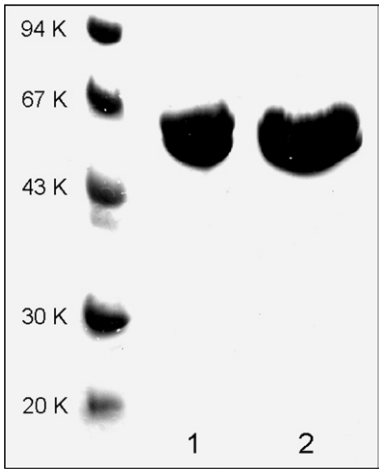

Fig. 2. Coomassie blue-stained SDS–PAGE of molecular mass markers (left lane), *N*-iminylamidase (lane 1, 25 μg) and purified mammalian imidase (lane 2, 35 μg). SDS–PAGE (12%) of purified *N*-iminylamidase (56 K) and protein standards (from Pharmacia) with size noted in kilodaltons: phosphorylase b (94 K), bovine serum albumin (67 K), ovalbumin (43 K), carbonic anhydrase (30 K), and soybean trypsin inhibitor (20.1 K).

shown). This result was based on the assumptions of similar shape and partial specific volume, as compared with globular protein, thyroglobin (669 K), ferritin (440 K), catalase (232 K), and aldolase (158 K) as molecular weight standards (Pharmacia). Thus, we reported here the enzyme was a tetramer of subunits of similar size that were not covalently joined.

Protein concentration

The protein concentration of enzyme solution was determined by A_{280} or BCA protein assay (Pierce, USA) using bovine serum albumin as a standard. For the homogeneous *N*-iminylamidase, 1 U of A_{280} equals 1.17 mg/ml *N*-iminylamidase based on the BCA protein assay.

Results and discussion

The goal of this project was to search for an enzyme responsible for a new biochemical reaction in mammal. This amidase, with *N*-iminylamidase activity, was first reported here (Table 1). Although considerable progress has been made in recent years in the study of the amidohydrolase superfamily [4], several fundamental questions concerning their reaction mechanism, substrate overlapping, specificity, and selectivity remain poorly understood. The present study made an attempt to address these important issues. As a first step toward achieving this goal, we have started investigating the existence of various reactions of amidohydrolase especially in mammal.

Discovery and purification of N-iminylamidase in pig liver

3-Iminoisoindolinone was identified as a substrate by a purified *N*-iminylamidase. *N*-Iminylamidase was purified approximately 450-fold with 11% yield from a soluble extract of pig liver by heat treatment, precipitation of ammonia sulfate, octyl-Sepharose, DEAE–Sephacel, and chelating-Sephacel column chromatographies (Table 1). Purified *N*-iminylamidase was ascertained to be homogeneous from the appearance of a single protein band on SDS–PAGE (Fig. 2, lane 1). Further evidence of its purity was provided by gel filtration (Sephacryl 300, Amersham–Pharmacia), giving a quite symmetrical protein absorption peak concomitant with 3-iminoisoindolinone activity.

Regiochemistry of N-iminylamidase

Hydrolysis of 3-iminoisoindolinone by *N*-iminylamidase was selective at only C–N bond near ketone (Fig. 1, lane I). The reaction of *N*-iminylamidase was monitored by the HPLC (Fig. 3) and ^{13}C NMR spectrometry (Fig. 4). Extra chemical shifts and peaks were produced following this enzymatic reaction with 3-iminoisoin- dolinone as shown in Figs. 3 and 4. Both of these results indicated that only one compound could be produced following the addition of this enzyme. HPLC analysis indicated that a more hydrophilic product was formed. The chemical shifts of the substrate and product were indistinguishable according to the program of ^{13}C NMR spectrometry prediction (CS ChemDraw Pro). Valuable information can still be obtained. Putative product III shown in Fig. 1 did not fit the ^{13}C NMR spectrometry (Fig. 4C), because its symmetric structure would have given only four ^{13}C chemical

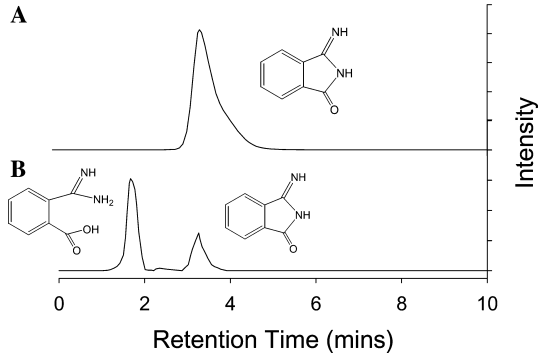

Fig. 3. HPLC analysis of 3-iminoisoindolinone as substrate of *N*-iminylamidase. 3-Iminoisoindolinone and its hydrolyzing product were separated and detected by HPLC with a 5 μm (250 mm) pre-packed LiChrospher 100 RP-18 column (Merck, USA) and were detected at 310 nm with a UV–vis detector using a D-7000 HPLC system (Hitachi, Japan). The separation was achieved in an isocratic eluent at a flow-rate of 1 ml/min. HPLC mobile phase was 50% water and 50% acetonitrile. (A) 3-Iminoisoindolinone (2 mM) with phosphate buffer (10 mM, pH 7.0). (B) After adding 20 μg *N*-iminylamidase to initiate the reaction in (A) for 2 h. This result demonstrated only one product could be found.

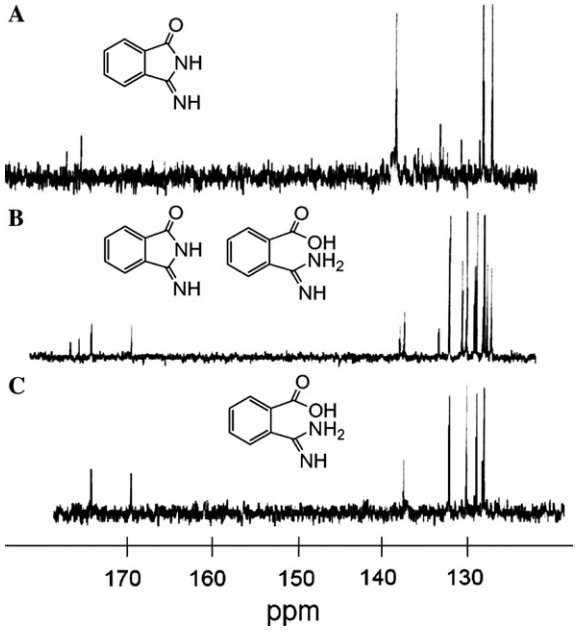

Fig. 4. Analysis of 3-iminoisoindolinone as substrate of *N*-iminylamidase by ^{13}C NMR. The ^{13}C NMR spectra were recorded on a Varian Unityinova 500 NMR spectrometer (Varian, USA) operating at a frequency of 125.7 MHz. Samples were placed in 5 mm NMR tubes, and the spectra were recorded at room temperature. A pulse of 4.6 μs was used with an acquisition time of 0.508 s. The spectral width was set to 36.53 kHz, and 37K data points were recorded for everyone free induction decay. (A) 3-iminoisoindolinone (2 mM) with phosphate buffer (10 mM, pH 7.0). (B) After adding 20 μg *N*-iminylamidase to initiate the reaction in (A) for 2 h. (C) After adding 20 μg *N*-iminylamidase to initiate the reaction in (A) for 4 days.

shifts. LC/MS measurement was found useful to determine the structure of the product (Fig. 5). Possible products (Fig. 1) were compared with the *m*/*z* value 165, 148, and

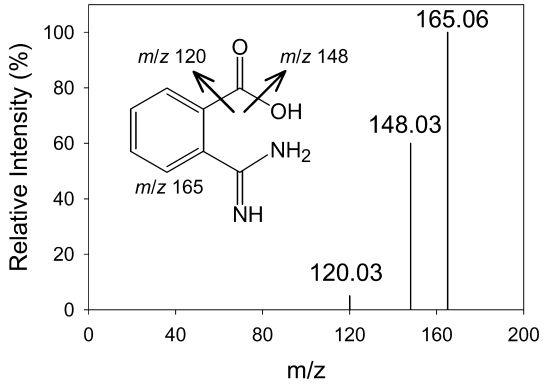

Fig. 5. Analysis of the product from the hydrolysis of 3-iminoisoindolinone by LC/MS. The product from the hydrolysis of 3-iminoisoindolinone was detected by LC/MS. Additional details were under Materials and methods.

120 of LC/MS measurement, only *o*-methyldiamine-benzoic acid was consistent with this result (Fig. 5). Both ^{13}C NMR spectrometry and LC/MS results determined that *o*-methyldiamine-benzoic acid was the only possible product of the *N*-iminylamidase-catalyzing reaction.

Substrate specificity and kinetic properties of N-iminylamidase

In the standard assay condition described above, the purified *N*-iminylamidase showed high catalytic efficiency toward the hydrolysis of 3-iminoisoindolinone as compared to the use of other substrates. The K_m and V_{max} values were calculated from the double reciprocal of Lineweaver–Burk plots using the program of Sigma-Plot were 2.5 ± 0.49 (mM) and 7.6 ± 1.1 (μmol/min/mg), respectively (Fig. 6). Other amide-like compounds were also tested. The following compounds were not active toward *N*-iminylamidase: barbituric acid (Fig. 7A), dihydrooro-

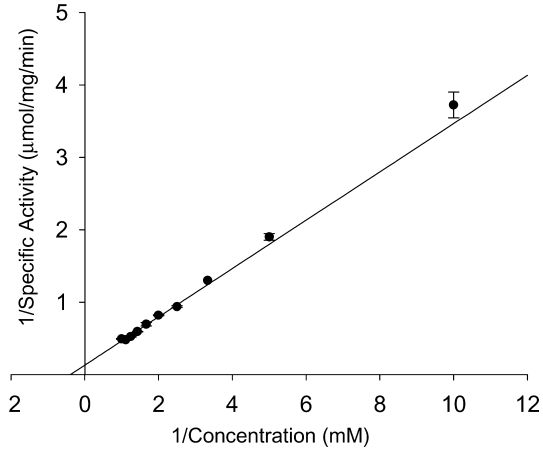

Fig. 6. The activity of *N*-iminylamidase by using 3-iminoisoindolinone as a substate. Kinetic study on *N*-iminylamidase for 3-iminoisoindolinone was carried out by using nine measurements determined at substrate concentration range from 0.1 to 1.0 mM. Additional details were described under Materials and methods.

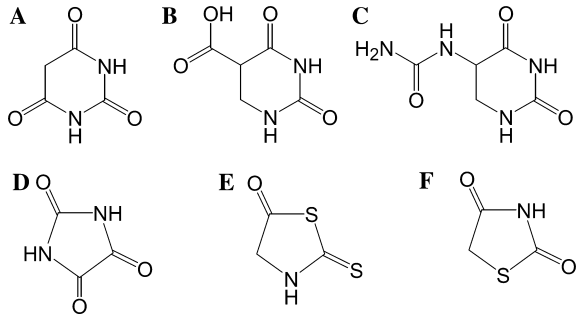

Fig. 7. Typical substrates of some cyclic amidase were inactive for pig liver *N*-iminylamidase. (A) Barbituric acid (barbiturase); (B) dihydroorotate (dihydroorotase); (C) allantoin (allantoinase); (D) parabanic acid; (E) rhodanine; and (F) 2,4-thiazolidinedione are substrates of *Blastobacter* sp. imidase.

tate (Fig. 7B), allantoin (Fig. 7C), parabanic acid (Fig. 7D), rhodanine (Fig. 7E), and 2,4-thiazolidinedione (Fig. 7F) which are substrates of barbiturase [6], dihydroorotase [16], allantoinase [17], and *Blastobacter* sp. imidase [9], respectively. Compounds were judged as inactive (non-substrate) toward *N*-iminylamidase in the following condition: 1 mg pig liver *N*-iminylamidase was mixed with the tested compounds (0.5–100 mM) at pH 7.9 (100 mM tris-buffer) for 1 h and the change of spectra of the compounds was not observed. These results indicated that pig liver *N*-iminylamidase catalyzed a new reaction and could not be classified or identified as barbiturase (EC 3.5.2.1), dihydroorotase (EC 3.5.2.3), allantoinase (EC 3.5.2.5), and *Blastobacter* sp. imidase (EC 3.5.2.16). Other compounds tested as non-substrate included uracil, pyromellitic diimide, phthalic anhydride, 5-bromouracil, 2,3-naphthalene-dicarboximide, 1,8-naphthalimide, 3,4,5,6-tetrachlorophthalimide, 4-amino-1,8-naphthalimide, 2*H*-1,3-benzoxazine-2,4 (3*H*)dione, biuret, bemegride, 5,5-dimethyl-hydantoin, uridine, 5-ethyl-methyl- hydantoin, parabanic acid, rhodanine, 2,4-thiazolidinedione, *N*-methylmaleimide, *N*-cyclohexylmaleimide, and oxindole.

Although a variety of heterocyclic compounds were tested and found inactive as described above, interestingly, pig liver *N*-iminylamidase was active with typical substrates, such as phthalimide [11], dihydrouracil [18], and maleimide [12], of mammalian imidase (EC 3.5.2.2). Their K_m values were 0.98 ± 0.07, 0.039 ± 0.012, and 15 ± 3 (mM), and V_{max} values were 27 ± 1, 0.16 ± 0.01, and 115 ± 8 (μmol/min/mg), respectively, catalyzed by *N*-iminylamidase. The K_m for phthalimide and maleimide under the same assay condition has previously been reported to be 1 and 13 mM with pig liver imidase [12]. These results were very similar to those of the enzyme purified here.

Acid–base dependence of N-iminylamidase

The pH profiles of V_{max}, K_m or V_{max}/K_m for pig liver *N*-iminylamidase determined in the pH range of 6.0–10.0 are presented in Fig. 8. The pK_a of *N*-iminylamidase was 7.41 ± 0.21, 5.69 ± 0.71 or 7.53 ± 0.07 for V_{max}, K_m or V_{max}/K_m, respectively. The activity of *N*-iminylamidase was pH-dependent (Fig. 8). The affinity of *N*-iminylamidase for 3-iminoisoindolinone was also pH-dependent, especially observed below pH 7. The pH profile of catalytic efficiency (V/K) for *N*-iminylamidase was highly similar to the pH profile of V_{max} of *N*-iminylamidase (Fig. 8C). A general-base catalyzed reaction of the hydrolysis of 3-iminoisoindolinone was expected according to the pH effects on *N*-iminylamidase.

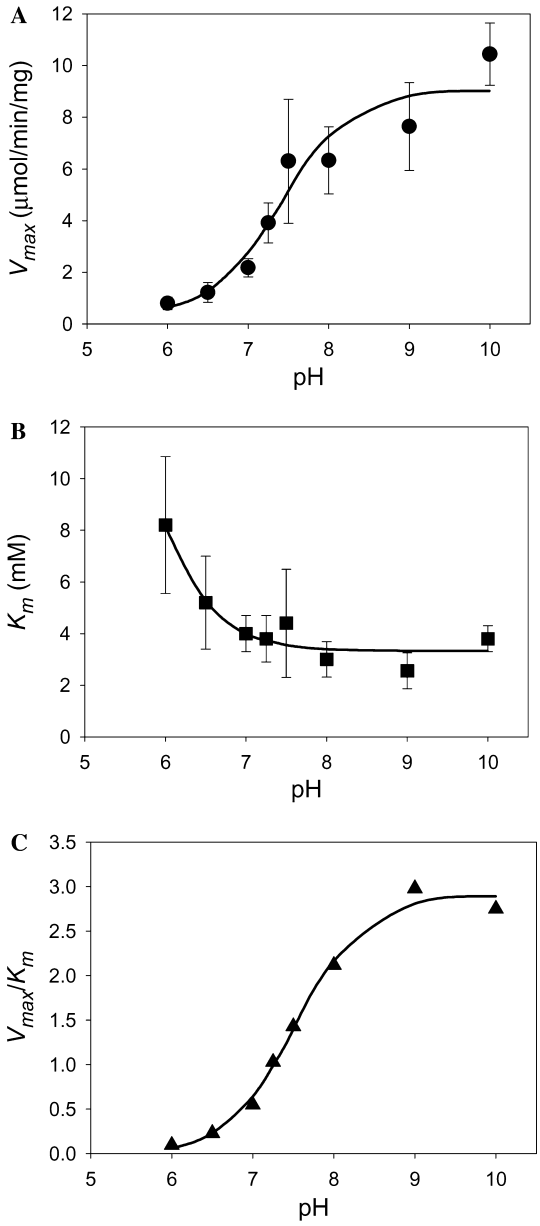

Fig. 8. The pH profiles of *N*-iminylamidase. The kinetic constants at various pHs were calculated and shown as plots of K_m (■), V_{max} (●), V/K (▲).

N-iminylamidase was identified as mammalian imidase

Of specific interest was the finding that this isolated enzyme appeared to be identical to mammalian imidase [11], an amidohydrolase catalyzing a large number of cyclic and linear imides. The purification procedures of *N*-iminylamidase were very similar to those of mammalian imidase [19], except for the alcohol treatment that was also used for the purification of bacterial hydantoinase [20]. Further experiment showed that two enzyme activities were purified concomitantly and that, in each case, only a single protein was apparent at each step of purification (data not shown). Protein absorption peak highly correlated with 3-iminoisoindolinone activity in a gel filtration experiment for imidase (data not shown). The substrate specificities of this enzyme were nearly identical to that of mammalian imidase described above. Indeed, mammalian imidase in this study was also purified to electrophoretic homogeneity (Fig. 2, lane 2) by the published protocol [19] to test the *N*-iminylamide activity of mammalian imidase. As expected, pig liver imidase was able to catalyze 3-iminoisoindolinone with high activity, and therefore we report here mammalian imidase could use 3-iminoisoindolinone as a substrate. Based on this information, we put forward an extended substrate range of mammalian imidase (Fig. 9) including 3-iminoisoindolinone. 3-Iminoisoindolinone was also found to be a substrate of bacterial hydantoinase (Huang and Yang, unpublished data). Bacterial hydantoinase was also highly active toward allantoin (unpublished results); however, mammalian imidase was inactive when allantoin was used as a substrate (Fig. 7C).

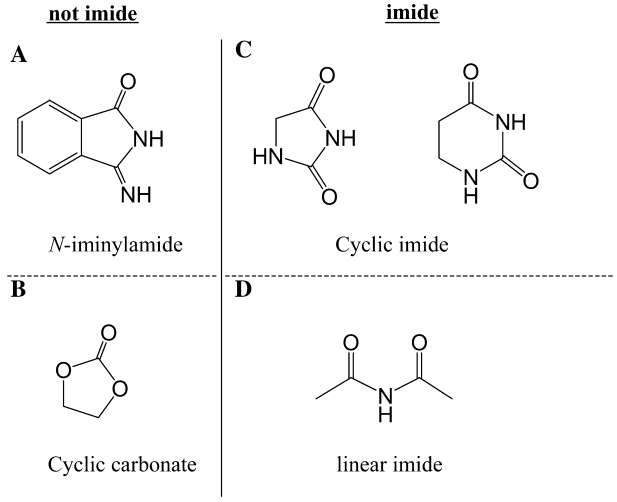

Fig. 9. Substrate range of mammalian imidase.

Proposed reaction mechanism of N-iminylamidase using 3-iminoisoindolinone as substrate

Detailed sketch for the chemicoselectivity of substrate of *N*-iminylamidase is shown in Figs. 3–5, based on the results of HPLC, ^{13}C NMR and LC–MS analysis. On the basis of the kinetic study of pig liver *N*-iminylamidase (Fig. 8), the reaction mechanism is proposed in Fig. 10.

The metal–water complex as indicative of the formation of the metal–hydroxide complex has been proposed to be responsible for the hydrolysis of a variety of imides [21]. However, the kinetic study of metal ion-replaced mammalian imidase did not support the metal–hydroxide complex to be important as a nucleo-

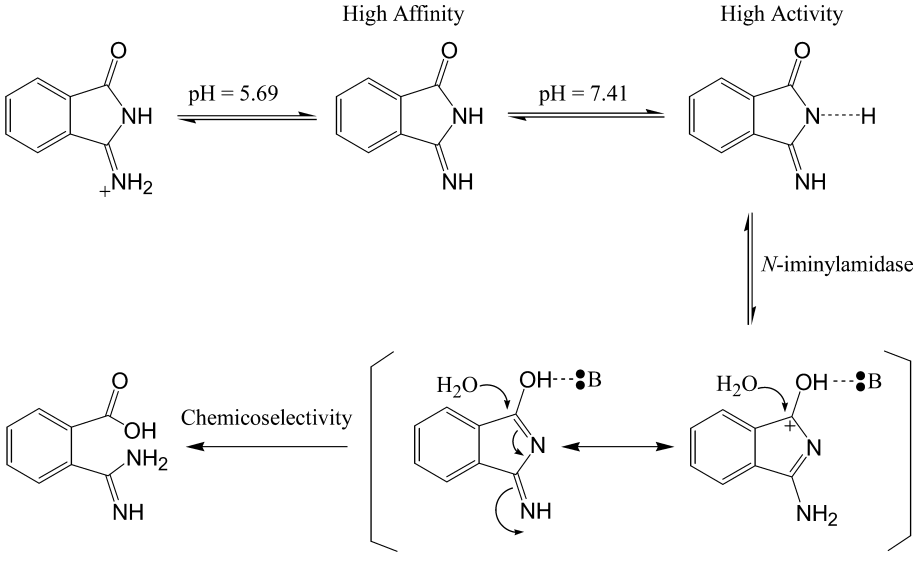

Fig. 10. Proposed reaction mechanism of *N*-iminylamidase. According to the pH profiles shown in Fig. 8, we proposed that the removal of the amide proton of the *N*-iminylamide was the driving force to promote the ring-opening process of 3-iminoisoindolinone catalyzed by pig liver *N*-iminylamidase. This reaction was chemicoselective by the evidences from Figs. 3–5. We sketched the mechanism of *N*-iminylamidase action based on these results.

phile, because the difference in pK_a values among metal-reconstituted mammalian imidases was not observed [22]. The pH profiles of *N*-iminylamidase activity (Fig. 8) significantly differed from that of imidase when other imide substrates were used [22]. When phthalimide was used as a substrate catalyzed by mammalian imidase, the pH profile was bell-shaped and the pK_a value was around 6.0 and 9.0 (zinc form; adapted from [22]). In this study, hydrolysis of 3-iminoisoindolinone gave only one pK_a at around 7.5. Indeed, pH activity relationships and correlation with the pK_a of the imidase substrates have been found previously [11]. A mechanism suggests that the removal of the imide proton forces the C4–N3–C2–N1 of dihydrouracil (Fig. 9C, right) toward a greater planarity in accommodating the delocalized electrons. This was proposed to be the driving force for imide hydrolysis catalyzed by mammalian imidase [11].

These seemingly contradictory data can be reconciled by recent studies. The reaction mechanism of the hydrolysis of 3-iminoisoindolinone catalyzed by mammalian imidase presented here was also consistent with previous report [11] by use of non-imide substrate examined, and reconfirmed that the rate-limiting step of mammalian imidase catalyzed is the removal of the imide proton or, in this case, the removal of the *N*-iminylamide proton. This mechanism explains the fact that unlike that of peptidases or proteases, mammalian imidase does not catalyze hydrolysis of amide or ester [11]. At present, no structural data are available for mammalian imidase or eukaryotic imidase. Recently, the crystal and its preliminary analysis of mammalian imidase have been obtained [23]. It would be expected that the structural basis of the differences in metal content [22,24–27], thermostability [12,28], and substrate specificity [9,11–13,24,29] among imidases from bacteria, slime mold, plant, insect, or mammalian will be analyzed in the near future.

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