Identification, Purification, and Characterization of a Thermophilic Imidase from Pig Liver

Tian-Mu Su and Yuh-Shyong Yang[1]

Institute of Biological Science and Technology, College of Science, National Chiao Tung University, Hsinchu, Taiwan, Republic of China

This study investigates thermophilic imidase activity of the liver. We demonstrate that imidase catalyzes the hydrolysis of imides at a temperature substantially higher than that of its native environment. Then, a thermophilic imidase is purified to homogeneity from pig liver, and its thermoproperties are studied. About 2500-fold of purification and 15% yield of imidase activity are obtained after ammonium sulfate precipitation, octyl, DEAE, chelation, and gel filtration chromatography. While avoiding heat treatment for the protein purification, this study also indicates that only one enzyme is responsible for the imidase activity. This homogenous enzyme prefers to catalyze hydrolysis of imides at above 60°C rather than at the body temperature of a pig. Although stable at below 50°C, imidase quickly loses its activity at above 65°C. Thus, the temperature effect on imidase activity is limited mainly by its thermostability. Substrate specificity of imidase is also temperature dependent. Our results demonstrate that the hydrolysis of physiological substrates is the most temperature dependent and that of hydantoins is the least temperature dependent. When increasing the reaction temperature from 25 to 60°C, specific activities increase 50- and 60-fold for dihydrouracil and dihydrothymine, respectively. The temperature effect on the K_m and V_{max} of imidase is substrate dependent.   © 2000 Academic Press

Imidase, due to its broad substrate specificity, is also known as dihydropyrimidinase (EC 3.5.2.2), hydantoinase, dihydropyrimidine hydrase, and dihydropyrimidine amidohydrolase. *In vitro* data indicate that imidase prefers many xenobiotics over physiological substrates (1). A recent finding further extends the substrate specificity of imidase to catalyze the hydrolysis of organic cyclic carbonates (2). This study describes the unexpected thermophilicity of imidase from an animal in which the optimal temperature for catalysis is more than 20°C higher than that of its native source.

Imide-hydrolyzing enzymes have been obtained in a homogeneous form from *Pseudomonas* (3,4), *Bacillus* (5), *Agrobacterium* (6), bovine liver (7,8), calf liver (9), pig liver (10), and rat liver (1,11). Imidase from animal sources are commonly referred to as dihydropyrimidinase since its only known physiological function is the hydrolysis of dihydrouracil and dihydrothymine (12). Recent investigations have discovered that a variety of proteins have a significant sequence homology but lack a known functional relationship with imidase (13–16). Several more sequences are found from *Rattus norvegicus* (GenBank Accession Nos. 3122017, 1351260, 3122038, and 3122037), *Mus musculus* (GenBank Accession Nos. 3122030, 3122040, 3122052, and 3122044), *Xenopus laevis* (GenBank Accession No. 3122041), *Box taurus* (GenBank Accession Nos. 3122018), and *Gallus gallus* (GenBank Accession No. 3122036). Even though previous investigators contend that some of these proteins are important for neural growth and development (13,16), they do not exhibit imidase activity. However, in addition to its wide spectrum of substrates, imidase activity has a measurement condition that strongly depends on the substrates used (1). Thus, the possibility of imidase activity cannot be ruled out until different conditions and substrates have been tested.

Matsuda *et al.* compare cDNAs of the rat liver imidase (15) and two other bacterial enzymes, *Bacillus stearothermophilus* (18) and *Pseudomonas putida* (19), and reveal a significant sequence homology (15). The cDNAs of imidases from yeast (20), *Pseudomonas* NS671 (21), and *Agrobacterium* (6) are also known.

[1] To whom correspondence should be addressed at Institute of Biological Science and Technology, National Chiao Tung University, Hsinchu 30050, Taiwan. Fax: 886-3-5729288. E-mail: ysyang@cc.nctu.edu.tw.

Analyzing the sequences of these enzymes provides valuable information regarding the function and the amino acid sequence. Interestingly, an enzyme from a bacterium is closely related to the enzyme from an animal, and the enzyme from thermophile, *B. stearothermophilus,* also closely resembles the enzyme from mesophile (rat and *Pseudomonas putida*). The question is whether not the thermoproperty is also similar for imidases from mesophile and thermophile. A recent investigation examined a thermophilic D-hydantoinase isolated from a mesophilic *Bacillus* sp. AR9 (17), indicating a substrate specificity similar to that of imidase. However, this enzyme has not been purified to homogeneity; thus, comparing its property with the known mesophilic imidase is impossible.

Our preliminary studies demonstrated that imidases from a mammalian source contains thermophilic properties similar to those of imidase from mesophilic and thermophilic bacteria (18,19). Despite the relative ease in finding mammalian proteins that are stable at an elevated temperature, to our knowledge, no work has been reported on a mammalian enzyme preferring a reaction temperature much higher (>20°C) than that of its native environment. Therefore, this study attempts to confirm our preliminary finding that thermophilic enzymes exist in a mammalian source, so we have purified a pig liver imidase to homogeneity and studied its thermoproperty. Whether or not one or more enzymes are responsible for all the different activities has also been examined.

MATERIALS AND METHODS

Materials

Rat liver was provided by Dr. T.-T. Kan (Institute of Toxicology, National Taiwan University Medical School). Dr. C.-F. Do (Pig Research Institute, Taiwan) provided both young (2 and 14 days old) and mature (4 months old) pig livers. The livers were frozen at −20°C immediately after being removed from the animals, then stored at −80°C. Mature pig livers were also obtained from the supermarket (frozen or fresh at around 4 to 8°C) or obtained fresh (storage at room temperature for several hours) from a traditional market and frozen at −80°C. Liver acetone powders from porcine, horse, cows, mice, sheep, rat, and guinea pig were purchased from Sigma (U.S.A.). Octyl Sepharose CL-4B, DEAE[2] Sephacel, chelating Sephacel (fast flow), a gel filtration calibration kit, and Sephacryl S-300 (HR) were purchased from Pharmacia. Bis-Tris

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[2] Abbreviations used: PMSF, phenylmethylsulfonyl fluoride; Tris–HCl, tris(hydroxymethyl)aminomethane hydrochloride; DEAE, diethylaminoethyl; EDTA, (ethylenedinitrilo)tetraacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Bis-Tris propane, 1,3-bis(tris(hydroxymethyl)methylamino)propane; Mes, 4-morpholine ethanesulfonic acid.

propane, dihydrouracil, dihydrothymine, PMSF, and phthalimide were purchased from Sigma. A BCA protein assay kit and albumin standard were obtained from Pierce (U.S.A.). EDTA, sodium chloride, sodium hydroxide, Tris–HCl, and zinc acetate were obtained from J. T. Baker (U.S.A.). Glutarimide, hydantoin, and succinimide were purchased from Aldrich (U.S.A.). All other chemicals were obtained commercially at the highest purity possible.

Preparation of Liver Extracts

About 3 g of liver was mixed with 3 vol of a pH 8 buffer including 50 mM Tris–HCl, 2 mM EDTA, and 1 mM PMSF (buffer A). The mixture was kept in an ice-cold environment and homogenized in a Waring blender for two periods of 30 s with a 1-min cooling interval between each blending. The suspension was centrifuged for 30 min at $10,000g$, and the resultant supernatant liquid was centrifuged for 120 min at $47,000g$. The final supernatant liquid was used for enzyme assay. The same procedures were used for extracting imidase for purification except that a larger amount of pig liver was used.

Imidase from the liver acetone powder was extracted by mixing 50 mg of the acetone powder with 500 μl solution including 0.2 M Tris–HCl at pH 7 and was gently stirred for 10 min. The supernatant was obtained by centrifugation. The same procedure was repeated twice to ensure that most of the imidase activity was dissolved in the solution; then the supernatant was heated at 60°C for 20 min. Precipitation was produced following heat treatment and was removed by centrifugation.

Protein Concentration

The protein concentration was determined by A_{280} or by BCA protein assay (Pierce, U.S.A.) using bovine serum albumin as a standard. For the homogeneous imidase, 1 unit of A_{280} equals 0.8 mg/ml based on the BCA protein assay.

Enzyme Assay

A rapid spectrophotometric assay (1) was used as the standard assay which meant the decrease in absorbency at 298 nm was measured upon hydrolysis of phthalimide as the substrate at 25°C. To start the reaction, an appropriate amount of liver extract (about 10 μl) or purified imidase was added into 1 ml solution, containing 1 mM phthalimide and 100 mM Bis-Tris propane at pH 7.0. Under these conditions, a change in A_{298} of 2.26 represents the hydrolysis of 1 μmol of the substrate. A unit of activity means the amount of enzyme catalyzing the hydrolysis of 1 μmol of phthalimide/min. The hydrolysis of imide was monitored with

a UV/VIS spectrophotometer (GBC 918) in a Peltier effect chamber maintained at the desired temperature with a thermostat circulator. The change in temperature does not appear to affect the extinction coefficient of phthalimide. However, the pH value of the same reaction mixture may vary at different temperatures. Thus, pH of the reaction mixture was determined at the temperature used to measure the imidase activity.

Purification of Imidase

Fresh pig livers were cut into pieces (about 80 g each) and frozen at $-80°C$ for less than 3 months before use. 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 (Bio-Rad Biologic) and an Amicon (UK) or Pharmacia column were used for column chromatography.

Step 1: Extract.   Imidase was extracted from frozen pig liver (270 g) and 540 ml buffer A; all other procedures were carried out as described above.

Step 2: Salting out.   Ammonium sulfate (equivalent to 30% of saturation) was slowly added to the enzyme extract and stirred for 60 min. The suspension was centrifuged at $20,000g$ for 30 min to remove precipitate and an additional ammonium sulfate was added into a supernatant fluid to 50% saturation. After gentle stirring for 60 min, the resultant precipitate was collected by centrifugation ($20,000g$, 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 ($20,000g$, 30 min). The dissolution of enzyme precipitate was repeated once to ensure that all the imidase activity was collected in the solution.

Step 3: 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 to 0 M ammonium sulfate using 350 ml each of buffer B and buffer B minus ammonium sulfate. Active fractions (approximately 8 ml each) were pooled (fractions 40–55). The active fractions were concentrated and desalted by ultrafiltration (Amicon, YM100 membrane), and the buffer solution was exchanged with buffer C (10 mM Tris–HCl, pH 8, 1 mM PMSF, and 2 mM EDTA).

Step 4: DEAE sephacel.   The desalted enzyme solution was loaded into a column of DEAE-Sephacel (2.6 × 15 cm, previously equilibrated with buffer C). The enzyme was eluted with the linear salt gradient from 0 to 0.3 M NaCl with buffer C and buffer C plus 1 M NaCl (total volume 350 ml). The pooled active fractions (frac-

TABLE 1

Thermophilicity of Imidase from Mammalian Livers

Source of crude liver extract	Specific activity (μmol/min g)		Ratio of specific activity (50°C/25°C)
	25°C	50°C	
From acetone powder			
Porcine	11.1 ± 0.35	55.0 ± 0.10	5.0
Horse	22.9 ± 0.24	66.4 ± 0.49	2.9
Bovine	6.1 ± 0.04	15.1 ± 0.28	2.5
Rat	1.4 ± 0.34	3.0 ± 0.07	2.2
Sheep	0.8 ± 0.09	1.8 ± 0.05	2.2
Guinea pig	0.27 ± 0.12	0.45 ± 0.15	1.7
Mouse	—[a]	0.11 ± 0.04	—
From frozen liver			
Pig	2.0 ± 0.07	13.3 ± 0.08	6.7
Mouse	0.84 ± 0.02	3.8 ± 0.11	4.5

Note. Enzymatic activity was measured as described under Materials and Methods for standard assay at pH 7. The pH of the buffers were determined at 25 or 50°C, respectively. Each data point was the average of at least three measurements ± standard deviation. Spontaneous hydrolysis of phthalimide at 50°C was observed ($\Delta A_{298} = 0.0076/min$ or 6.9 nmol/min) under the standard assay conditions and was subtracted as background. Extracts from acetone powder and liver were obtained as described under Materials and Methods for enzyme purification. The extract was heated at 60°C for 20 min following by centrifugation to remove precipitate.

[a] The activity was too small to measure.

tions 37–46, 4 ml/fraction) were concentrated and desalted by ultrafiltration, and the buffer system was replaced with 20 mM phosphate, 1 mM PMSF, and 0.5 M NaCl at pH 7 (buffer D).

Step 5: Chelating sephacel.   The enzyme solution in buffer D was applied to a column (1.6 × 10 cm) of chelating Sephacel (fast flow) that was treated with 1 gel volume of 0.2 M zinc acetate and then equilibrated with buffer D. 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 200 ml). The active fractions were pooled and concentrated to 1 ml by ultrafiltration.

Step 6: Sephacryl.   The concentrated imidase solution (1 ml) was loaded into a column (1 × 97 cm) of Sephacryl S-300HR which had been equilibrated with buffer A. Homogeneous imidase was eluted with buffer A at the speed of 0.5 ml/min. Active fractions were pooled, concentrated by ultrafiltration, and frozen at $-80°C$ for later analysis.

Protein Purity and Molecular Weight Determination

The purity and subunit molecular weight of imidase were determined by SDS–PAGE. Gel electrophoresis was performed in a Mini-Protein II Electrophoresis Cell (Bio-Rad, U.S.A.). A 10 or 12% acrylamide gel with

TABLE 2

Summary of Purification of Pig Liver Imidase

Step	Volume (ml)	Total activity (μmol/min)	Total protein (A_{280})	Specific activity (μmol/min A_{280})	Yield (%)	Fold of purification
Extract	510	222	71810	0.003	100	1
Salt precipitation	128	258	4250	0.06	116	20
Octyl Sepharose	41	120	654	0.19	54	60
DEAE-Sephacel	9.4	93	118	0.79	42	256
Chelating Sephacel	38.5	47	8.4	5.6	21	1800
Gel filtration	7.8	34	4.4	7.7	15	2480

4% stacking gel was used with SDS according to the method of Laemmli (22). Protein was stained with Coomassie blue R-250 (National Diagnostics). Gel filtration (as described earlier for protein purification) was used to estimate the native molecular weight of imidase. Void volume was determined by Blue Dextran 2000, and molecular weight standards were used, which include thyroglobin (669K), ferritin (440K), catalase (232K), and aldolase (158K).

RESULTS

Table 1 summarizes the differences in imidase activity from liver extracts of mammals at room temperature and at a temperature exceeding the body temperature of the host. The liver extracts were heated at 60°C for 20 min and centrifuged before assaying for imidase activity. This procedure minimizes the precipitation of protein at an elevated temperature which may interfere with the absorption change at 298 nm. The heat treatment did not appear to affect imidase activity at either 25 or 50°C. This indicated that most of the imidase activity measured originates from heat-stable enzymes. Imidase activities of the liver increase about two- to sixfold when the temperature increases from 25 to 50°C. Data from Table 1 indicate that imidases from different animal sources may significantly differ in both quantity and property. This observation allows us to infer that thermophilic imidase may be best obtained from pig liver whose activity shows the most temperature dependency. Herein, various sources of imidase activity from pig liver were also tested. Gender does not significantly affect imidase activity. However, the specific activity of imidase (imidase activity/weight of liver) from a young pig liver (2 days old) is only about 10% of that from the livers of 2-week-old or older pigs. Little difference in imidase activities was observed from fresh or frozen pig liver before and after heat treatment.

Purification of Imidase from Pig Liver

Imidase from pig liver was purified about 2500-fold with 15% yield (Table 2) producing a homogeneous

protein according to the criteria of SDS–PAGE (Fig. 1). The heat-treatment procedure was deliberately avoided during the purification so that the possible existence of thermolabile imidase would not be inactivated. Previous investigations have confirmed that the heat treatment is quite useful in purifying other similar enzymes from rat (10), calf (8), and pig (9) livers. Omitting this procedure to purify imidase from rat liver required a tediously large number of procedures to remove the minor contaminants that were visible on SDS–PAGE (1). In this study, we have presented a purification scheme that does not rely on heat treatment and still obtains a reasonable yield. Since the temperature effect on imidase is of primary concern for this study, a homogeneous enzyme must be prepared without previously experiencing heat much higher than its original environment. Also, a different thermolabile imidase may be observed in this purification scheme, if it exists. Herein, only one imidase from pig liver was observed.
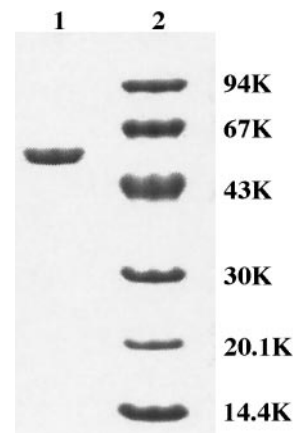

FIG. 1. SDS–PAGE (12%) of purified pig liver imidase and protein standards (from Pharmacia) with size noted in kilodaltons: phosphorylase b (94K), bovine serum albumin (67K), ovalbumin (43K), carbonic anhydrase (30K), soybean trypsin inhibitor (20.1K), α-lactalbumin (14.4K).
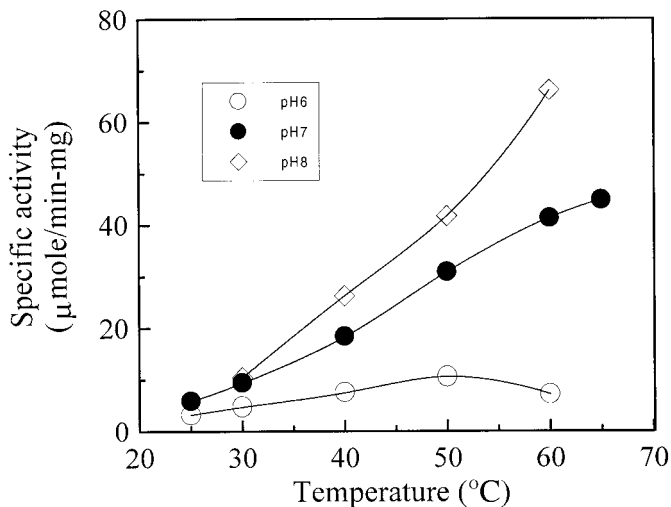
FIG. 2. Effect of temperature and pH on the activity of imidase. Reaction mixture as described under Materials and Methods for standard assay was equilibrated at desired temperature. Imidase (about 1.5 μg) was added to start the reaction. The pH of the solution (with 100 mM Bis-Tris) was determined at 25°C. As temperature increases from 25 to 60°C, the pH decreases from 6, 7, and 8 to 5.3, 6.3, and 7.3, respectively. Each data point is the average of at least three measurements, which differ by less than 10%.

Homogeneity and size of the thermophilic imidase SDS–PAGE in Fig. 1 shows the purity and molecular weight of imidase. The molecular weight for a single polypeptide chain is estimated to be around 51,000. Using thyroglobin (669K), ferritin (440K), catalase (232K), and aldolase (158K) as molecular weight standards, the molecular weight of imidase estimated by gel filtration is about 300K.

Thermophilicity and Thermostability of Pig Liver Imidase

According to Fig. 2, purified imidase from the pig liver exhibits thermophilicity. Imidase activity continues to increase with the elevation of temperature (up to 65°C) except for a reaction at pH 6. As shown in Figs. 3 and 4, the activity of imidase is limited by its thermostability and pH of the reaction condition at an elevated temperature. These figures reveal that the stability of imidase markedly decreases in an acidic or alkaline pH at 60°C (Fig. 3). At a pH around 7.5 to 8.5, imidase is stable at 50°C or at a lower temperature for over 2 h. The half-life of imidase at 60°C is about 35 min and decreases sharply to only 10 min at 65°C (Fig. 5). No enzyme activity can be observed when imidase is heated at 70°C for less than 10 min. This finding suggests that when pH is not a limiting factor, thermophilicity of imidase is limited mainly by its thermostability.

Effect of pH on the Thermostability and Thermophilicity of Imidase

The effects of pH and temperature on the activity and stability of imidase are shown in Figs. 3 and 4. Figure 3 reveals that at an acidic pH, imidase is stable at 40°C but less stable at a higher temperature (50 and 60°C). This observation accounts for the lesser thermophilicity of imidase at an acidic pH as shown in Fig. 2. At a lower temperature (below 40°C), imidase is stable over a wide pH range (Fig. 3). Therefore, according to Fig. 4, the stability of a protein does not influence its activity profile at 25°C. However, due to the instability of imidase at acidic and alkaline pHs (Fig. 3), the pH profile at 60°C (Fig. 4) is the combined result of imidase activity and stability. The pH profiles of imidase at 25 and 60°C deviate about 0.5 to 1 pH units as shown in Fig. 4. Notably, this shift of pH optimum is not attributed to the stability of imidase at extreme pHs, since imidase at 60°C is relatively stable under an alkaline condition up to pH 8.5 during the assay conditions (Fig. 3). Thus, the shift of pH optimum to a more acidic pH at elevated temperature indicates a change of imidase that affects its catalysis. Most likely, the pK_a of amino acid residue which is important for imide hydrolysis decreases at an elevated temperature.
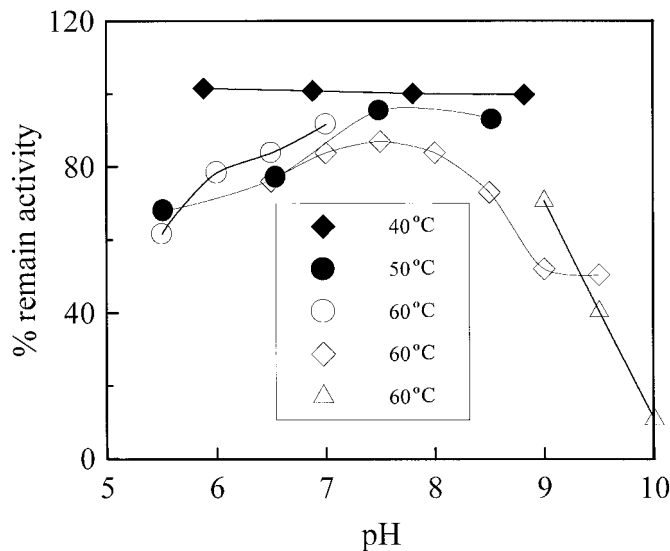

FIG. 3. Effect of pH and temperature on the stability of imidase. Imidase (7.6 μg) in 20 μl solution containing 100 mM Mes (pH 5.5–7.0), Bis-Tris (pH 6.5–9.5) or glycine (pH 9.0–10.0) was heated at 40, 50, or 60°C in a water bath for 10 min and then cooled in ice bath. The pH of the buffers was determined at each incubated temperature. Minor enzyme precipitate was observed during heating at 60°C and was removed by centrifugation. Heat treated imidase activity was determined by standard assay as described under Materials and Methods. Each data point is the average of at least three measurements, which differ by less than 10%.
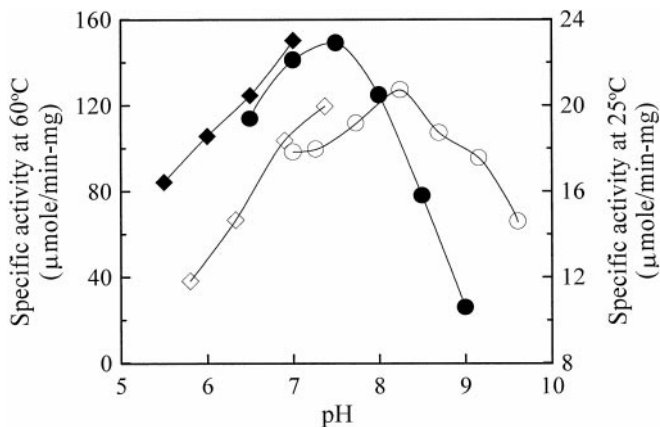
FIG. 4. The pH profiles of imidase activity at 25 and 60°C. The reaction mixture in suitable buffer was equilibrated at the desired temperature and then imidase (about 2.3 μg) was added to start the reaction. A circulated water bath and the Peltier temperature controller of GBC 918 spectrophotometer controlled the temperature. The pH of the mixture was determined at the reaction temperature. Mes and Bis-Tris (100 mM) were the buffers for pH 5.5–7.5 and 6.5–9.6, respectively. Open symbols denote activity at 25°C and closed symbols denote activity at 60°C. Each data point is the average of at least three measurements, which differ by less than 10%.

Effect of Temperature on the Substrate Specificity of Imidase

Three groups of substrates were examined to study the temperature effect on the substrate specificity of imidase as shown in Table 3. According to this table, temperature-dependent imidase activity varied significantly with different substrates. Toward the only two known natural substrates, specific activities of imidase exhibit the highest temperature dependency. Hydrolysis of dihydrouracil and dihydrothymine increases 50- and 60-fold, respectively, when the reaction temperature increases from 25 to 60°C. In contrast, imidase-catalyzed hydrolysis of hydantoins, i.e., a five-member ring analog of dihydrouracil, exhibits a rather insensitive temperature dependency. The imidase specificity for hydantoin and 1-methyl hydantoin increases only 2- and 3-fold, respectively, when the reaction temperature increases from 25 to 60°C. Thus, dihydrouracil, a poor substrate for imidase at 25°C, becomes a much better substrate at 60°C than hydantoins (Table 3). Phthalimide is used as a standard substrate, and its temperature dependency was studied extensively as summarized in Figs. 2, 3, and 4 and Tables 3 and 4. Tables 3 and 4 show that the temperature effect of glutarimide as a substrate of imidase resembles that of phthalimide. According to these tables, the effect of temperature on the substrate specificity of imidase appears to depend on the functional group and the structure of the substrates.

Effect of Temperature on the K_m, V_{max} and Catalytic Efficiency of Imidase

The changing reaction temperature may alter K_m and V_{max} of imidase, thereby affecting the specific activity as shown in Table 3. Table 4 indicates that the temperature effect on these constants is substrate dependent. Using hydantoins as a substrate at 25 or 60°C does not significantly affect its K_m and V_{max} of imidase. This is a sharp contrast to that of an imidase using dihydrothymine or phthalimide as the substrate. According to our results, V_{max} of imidase increases about 100-fold when the reaction temperature increases from 25 to 60°C; meanwhile, K_m also increases about 20- to 30-fold which is dependent on the substrates tested. Overall catalytic efficiency of imidase also increases when phthalimide or dihydrothymine is used as a substrate, but only around 2- to 4-fold. The large standard errors of the K_m and V_{max} data shown in Table 4 are attributed to the low solubility of hydantoins (in the millimolar range) and a high K_m value. Phthalimide is unstable at an elevated temperature. Spontaneous hydrolysis of 1 mM phthalimide at pH 7 is about 0.4 nmol/min at 25°C and elevates to 7 and 15 nmol/min when the temperature is increased to 50 and 60°C, respectively. All other substrates, including glutarimide, remain stable at their reaction condition within the time frame for enzyme assay.

The change of K_m of phthalimide and V_{max} of imidase is relatively mild from pH 5.5 to 8 as shown in Table 5.
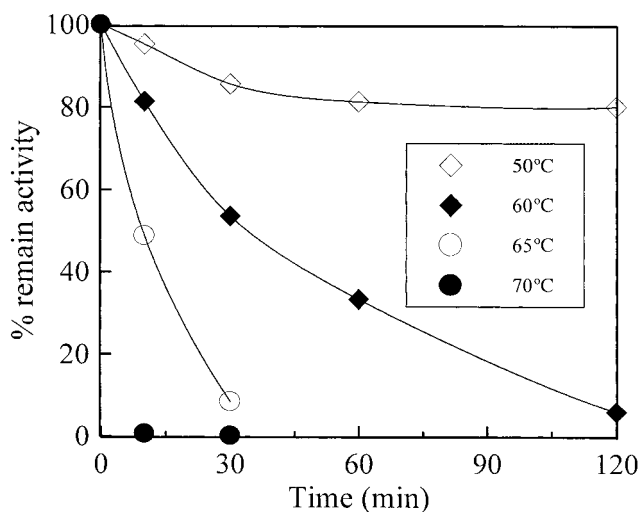

FIG. 5. The effect of temperature on the stability of imidase at pH 8. Imidase (5.2 μg) in 20 μl 100 mM Bis-Tris propane at pH 8 (determined at 25°C) was heated in water bath at the desired temperature. An aliquot of imidase was removed at the desired time and cooled in ice bath. The enzyme solution was centrifuged at 4°C to remove precipitate and assayed at 25°C as described under Materials and Methods for the enzyme standard assay. Each data point is the average of at least three measurements, which differ by less than 10%.

TABLE 3

Effect of Temperature on the Substrate Specificity of Imidase

Substrate	Concentration (mM)	pH	λ (nm)	ϵ (mM^{-1} cm^{-1})		Specific activity (μmol/min mg)		Ratio of specific activity (60°C/25°C)
				25°C	60°C	25°C	60°C	
Phthalimide	1	7	298	2.26	2.26	17.8 ± 0.02	141 ± 0.8	7.9
Glutarimide	4	7	220	0.54	0.54	0.31 ± 0.06	3.1 ± 0.04	10
Dihydrouracil	5	9	235	0.35	0.37	0.32 ± 0.03	16.1 ± 0.53	50
Dihydrothymine	5	9	235	0.38	0.39	0.28 ± 0.01	17.1 ± 0.53	61
Hydantoin	4	9	242	0.48	0.52	1.6 ± 0.18	3.6 ± 0.12	2.3
1-Methyl hydantoin	3	9	252	0.65	0.72	1.5 ± 0.10	4.6 ± 0.35	3.1

Note. Enzymatic activity was measured as described under Materials and Methods except that different pH and substrate concentrations were used. Specific activity given was the average of at least three measurements ± standard deviation. pH of the buffer (100 mM Tris–HCl) was determined at the reaction temperature (25 or 60°C). Extinction coefficients were determined experimentally by direct measurement with a spectrophotometer.

This result indicates that the effect of temperature on K_m of phthalimide and V_{max} of imidase may not correlate with the change of pK_a of imidase as implicated by the temperature affect on the pH optimum of imidase (Fig. 4).

DISCUSSION

A close examination of the imidase activities from various mammalian sources indicates that the optimal temperature far beyond their native environment may be a common feature for the imide hydrolyzing enzymes (Table 1). This observation correlates with recent data from molecular cloning which demonstrates that sequence homology of several imidases (or dihydropyrimidinase and related proteins) among several mammalians is very high (13–16). Several factors may affect the thermoproperty of an enzyme in extracts, such as the presence of inactivating proteases and other stabilizing or destabilizing molecules. In addi-

tion, the procedures for the preparation of imidase may also alter its enzymatic properties. Thus, in this study, our purification scheme for imidase is designed not only to avoid two of the conventionally used procedures for this type of enzyme, i.e., preparation of acetone powder and heat treatment, but also to purify imidase under a milder condition. This purification not only identifies a thermophilic enzyme, but also prepares an enzyme deemed appropriate for studying its thermoproperty. Our results found only one imidase responsible for all the imide hydrolysis and the thermophilicity.

A mammalian imidase was found not only to be heat stable, but it also prefers a much higher reaction temperature, over 25°C higher than its host's body temperature, for catalysis. Recent progress in the molecular cloning of several imide-hydrolyzing enzymes reported sequence similarity (30–40% homology in amino acid sequence) between the mammalian source (13) and

TABLE 4

Effect of Temperature and pH on the V_{max} and K_m and Catalytic Efficiency of Imidase

Substrates	pH	Temperature (°C)	K_m (mM)	V_{max} (μmol/min mg)	V_{max}/K_m
Phthalimide	7	25	0.18 ± 0.04	9.0 ± 0.7	50
	7	60	7.7 ± 6.4^a	970 ± 730^a	126
Hydantoin	9	25	9.0 ± 1.7^a	6.1 ± 0.9^a	0.68
	9	60	10.1 ± 6.9^a	6.5 ± 3.4^a	0.64
Dihydrothymine	9	25	0.015 ± 0.01	0.26 ± 0.01	17
	9	60	0.35 ± 0.09	25.9 ± 1.3	74

Note. Enzymatic activity was measured as described under Materials and Methods for the standard imidase assay except that different substrate, pH, and temperature were used. K_m and V_{max} were obtained by nonlinear regression ± standard error. The pH of buffer (100 mM Tris–HCl) was determined at the reaction temperature (25 or 60°C). Extinction coefficients were determined experimentally by direct measurement with a spectrophotometer.

[a] Large standard errors reflect the difficulty of obtaining enzyme activity at high substrate concentration due to the low solubility of substrates.

TABLE 5
Effect of pH on the V_{max} and K_m of Imidase Using Phthalimide as Substrate

pH	K_m (mM)	V_{max} (μmol/min mg)	V_{max}/K_m
5.5	0.75 ± 0.26	8.1 ± 1.5	11
6.0	0.47 ± 0.04	12.9 ± 0.5	27
7.5	0.48 ± 0.04	21.8 ± 0.8	45
8	0.68 ± 0.06	22.4 ± 1.0	33

Note. Imidase activities were measured as described under Materials and Methods at 25°C with different pHs. K_m and V_{max} were obtained by nonlinear regression ± standard error.

from one of the mesophilic bacterial enzymes (18). The bacterial enzyme also prefers a higher temperature (17) for imide hydrolysis than that of its native environment. Several other similar proteins (80–90% homology in amino acid sequence) with dihydropyrimidinase were also found from human (13) and rat (15). A related investigation cloned these dihydropyrimidinases and their related proteins to elucidate the possible relationship with neural growth (13) and its potential importance for the nervous system (16). However, the function of these proteins that contain a high amino acid sequence homology with imide-hydrolyzing enzyme remains unclear. Even for the dihydropyrimidinase, an enzyme that has been known since 1957 (23), investigations continue to discover its new substrates with different functional groups (1,2). The wide substrate spectrum of dihydropyrimidinase was found to include dihydrouracils, hydantoins, cyclic and linear imides (1), and cyclic carbonates (2). In this study, we have observed yet another property, thermophilicity, of this enzyme.

Regarding the reaction conditions and substrates used for catalysis, imidase is an enzyme of great diversity. In addition to its wide specificity with substrates of distinct structure and functional groups, imidase may catalyze hydrolysis of each substrate under a different optimal condition (1,2). By balancing thermophilicity and thermostability, the optimal temperature for imidase-catalyzed hydrolysis is around 60°C (Figs. 3 and 5). However, the intensity of temperature effect varies significantly with different substrates (Table 3). The spontaneous hydrolysis rate of phthalimide increases approximately twofold when the temperature is increased from 50 to 60°C (k_1, the first order rate constant of phthalimide hydrolysis at pH 7, is 7.1 and 15 μmol/min M, respectively, at 50 and 60°C). This observation is consistent with the fact that activity increases approximately twofold for every 10°C rise increase in reaction temperature. The temperature effect on enzymatic reaction (Tables 3 and 4) can be much more (for imides and dihydrouracils) or less (for

hydantoins) significant than that of the spontaneous reaction. Imidase-catalyzed hydrolysis of imides does not follow the rule for temperature dependence of simple reaction rates and thus largely depends on the change of the enzyme and not on the stability of the substrates.

At 65°C and above, imidase activity is limited mainly by its thermostability which subsequently depends on pH (Fig. 3). Below 60°C, imidase is relatively stable at a wide pH range (Fig. 3) during the assay time frame. In addition, although the pH of the reaction condition affects both K_m and V_{max}, it is not as extreme when phthalimide is used as a substrate (Table 5). A related investigation found that the V_{max} of dihydropyrimidinase is also pH independent between 6.5 and 10 (10). The temperature effect on K_m and V_{max} of imidase strongly depends on the substrate used (Table 4). The significant change of K_m and V_{max} (10- to 100-fold differences for phthalimide and dihydrothymine) as shown in Table 4 is mainly temperature dependent. However, the temperature effect of imidase does not apparently affect its hydrolysis rate of hydantoins (Table 4). The K_m of imidase with hydantoin as a substrate is already quite large at 25°C; no significant change can be observed for both K_m and V_{max} when the reaction temperature was increased from 25 to 60°C. For its large selection of substrates, an active site of imidase must accommodate a wide range of different structures and functional groups. Notably, elevating the temperature may further increase its flexibility, as shown by the higher K_m of imidase at a higher temperature for various substrates. We predict an even wider substrate spectrum for imidase at an elevated temperature.

Yang *et al.* (1) proposed a mechanism for imide hydrolysis based on the substrate specificity of imidase, stability of the substrates and their three-dimensional and resonance structure (24), and the correlation of pH profile and pK_a of the substrates (1). The hydrolysis of cyclic carbonate was found to fit well with the proposed mechanism (2). Removing the imide proton (N3 of dihydrouracil) produces an electron sink (N1) to facilitate a nucleophilic attack on the C4 carbon, thereby accounting for the resulting *N*-carbamoyl product. The pK_a of an imide may vary with the change of temperature, as shown by the differences in extinction coefficients shown in Table 3 at different temperatures. The increase in the extinction coefficient of hydantoin indicates that the pK_a of imide decreases so that more deprotonated form of hydantoin is formed at the alkaline pH. According to Fig. 4, the pH optimum of an imidase shifts to a more acidic pH at a higher temperature. This observation is consistent with the change of pK_a at a different temperature and may be analogous to the different pH profiles observed (1) by using different substrates with different pK_as.

Previous investigations have postulated that metals

(zinc, manganese, and cobalt) are involved in imide hydrolysis (8–10) and act as a general base. Magnesium was found to be important for rat liver imidase when dihydrouracil was used as a substrate. A related work proposed that magnesium is important for the release of the product (1). Results in this study demonstrate that magnesium is unnecessary for the hydrolysis of dihydrouracil which is catalyzed by pig liver imidase (data not shown). This proposed mechanism does not necessarily contradict with the one including metals (10) for the nucleophilic attack by a water molecule that may be facilitated by the metals.

The temperature effect on the imidase-catalyzed hydrolysis is dependent on the structure and functional group of substrates. The mechanism of imidase-catalyzed hydrolysis of imides is now under investigation.

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