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A novel cold-adapted imidase from fish *Oreochromis niloticus* that catalyzes hydrolysis of maleimide☆

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Abstract

In this paper we report the first comparative study of cold-adapted imidase (EC 3.5.2.2) from the fish (*Oreochromis niloticus*) liver and its thermophilic counterparts taken from pig liver and *Escherichia coli* (overexpressed recombinant hydantoinase from *Agrobacterium radiobacter* NRRL B1). Approximately 6000-fold purification and a 40% yield of fish imidase activity were obtained through ammonium sulfate precipitation, octyl, chelating, DEAE, and hydroxyapatite chromatography. This cold-adapted imidase was characterized by a specific activity 10- to a 100-fold higher than those of its thermophilic counterparts below room temperature (25 °C or lower) conditions but less stable at elevated temperatures (40 °C or higher). A less organized helical structure (compared to those of pig liver and bacterial imidases) was observed by circular dichroism. Furthermore, maleimide was first identified as a novel substrate of all imidases examined, and confirmed by HPLC and NMR analysis. These results constituted a first study to discover a novel cold-adapted imidase with surprising high activity. These findings might be also helpful for industrial application of imidase.

Psychrophilic organisms, either microorganisms, algae, invertebrates, or fish, live in permanently cold environments and have the ability to grow efficiently at temperatures close to the freezing point of water [1]. Nearly all biochemical reactions involved in cell metabolism are strongly temperature dependent. To cope with this vital temperature dependence of the enzyme reaction rates, these cold-adapted organisms synthesize heat-labile enzymes possessing high specific activities and catalytic efficiencies at low temperatures [2,3]. Heat-lability has been commonly related to the lack of selective pressure for stable proteins in cold environments, but this classical view has frequently been challenged. It is believed that the weak stability reflects a more resilient or flexible enzyme structure, ensuring a better accommodation of substrates and enhancing the fast conformational changes required by catalysis [4–7]. Thus, to obtain an enzyme possessing high activity, that from psychrophilic organisms might be considered first.

Imide-hydrolyzing enzymes [8,9] are industrial enzyme useds as a biocatalyst to produce the precursors of antibiotics [10], which possesses broad substrate specificities and catalyzes reactions in a wide range of temperatures [11]. Imide-hydrolyzing enzymes are widely distributed in living organisms and were first described as hydantoinase in the 1940s as occurring in plants and animals [12,13]. The natural substrates of the imidase isolated from calf liver are shown to be dihydropyrimidines and this enzyme has been named dihydropyrimidinase [14]. Although a component in the chain of pyrimidine catabolism, imidase is capable of serving a broader role that includes the detoxication of xenobiotics with imide functional group that ranges from linear imides to heterocyclic imides [9]. The substrate specificity of imidase further extends to catalyze the hydrolysis of organic cyclic carbonates [15]. Mammalian

☆ *Abbreviations:* 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; MES, 4-morpholine ethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; buffer A, 50 mM potassium phosphate, 1 mM PMSF, and 2 mM EDTA at pH 8; buffer B, 10 mM potassium phosphate, 1.5 M ammonium sulfate, 1 mM PMSF, and 2 mM EDTA at pH 7; buffer C, 10 mM potassium phosphate, and 1 mM PMSF at pH 8; buffer D, 20 mM potassium phosphate, 1 mM PMSF, and 0.5 M NaCl at pH 7.

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imidase was found to be thermophilic and its substrate specificity is temperature dependent [11]. Imidase has been obtained in a homogeneous form from *Pseudomonas* [16], *Bacillus* [17], *Agrobacterium* [18], bovine liver [19], calf liver [20], rat liver [9,21], and pig liver [11,22]. These enzymes possess broad and overlapping substrate specificity with subtle differences that have not been studied and compared in detail. Mammalian imidases are all tetrameric metalloproteins containing four tightly bound zinc atoms [19–21,23]. Some significant variations are found in the sequences and metal contents of imidases that catalyze similar reactions from different organisms. Recently, the crystal structures of hydantoinase from *Thermus* sp. [24] and *Bacillus stearothermophilus* [25] have been reported. They contain one bridged dimetal ion per enzyme subunit. More recently, the crystal and its preliminary analysis of a mammalian enzyme have been obtained [26]. It would be expected that the structural basis of the differences in metal content, thermostability, and substrate specificity among imidases from diverse sources will be available in the near future. Despite some variations that have been found from organism to organism, it is intriguing that all imidases tested for temperature dependency were found to be thermostable [11,17,18,20–22,27–32]. Psychrophilic imidase from any source has not been reported. In accord with our interest in the mechanism of imidase reactions [9,23] and properties [11], we sought to obtain an imidase that possesses different thermal activity. In this study, we first purified the cold-adapted imidase and compared its properties with those of its thermophilic counterparts.

Experimental procedures

Materials

The resistance of water used was more than 18 MΩ, which was purified by reverse osmosis followed by passage through a Millipore Reagent Water System (Millipore, Bedford, MA, USA). Octyl Sepharose CL-4B, DEAE Sephacel, chelating Sephacel (fast flow), and HiTrap desalting columns were purchased from Amersham Biosciences (Taiwan Branch). Bis–Tris propane, PMSF, and phthalimide were purchased from Sigma (USA). EDTA, sodium chloride, sodium hydroxide, Tris–HCl, and zinc acetate were obtained from J.T. Baker (USA). Hydroxyapatite Sephacel was purchased from Biosepra (USA). All other chemicals were obtained commercially at the highest purity possible.

Enzyme assays

A rapid spectrophotometric assay [9] was used as the standard assay. Briefly, the decrease in absorbancy at 298 nm was measured upon hydrolysis of phthalimide as the substrate at 25 °C. To start the reaction, the purified imidase was added into a 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. The hydrolysis of the substrate was monitored with a UV/Vis spectrophotometer (Hitachi U 3300).

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 imidase, 1 U of A_{280} equals 1.17 mg/ml imidase based on the BCA protein assay. Similar results were determined for all the three enzymes studied in this report.

Protein purification

Imidase taken from pig liver and hydantoinase (bacterial counterpart of imidase) taken from *Agrobacterium radiobacter* NRRL B1 were purified according to the published procedures [11,33]. Fish liver imidase was purified as described below.

Liver of fresh fish (*Oreochromis niloticus*) was frozen at −20 °C immediately after removal 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 the measurements taken at room temperature. An FPLC system (Pharmacia) and a Pharmacia column were used for column chromatography.

Step 1: extract. The imidase was extracted from the frozen fish liver (about 200 g) with a 400 ml buffer A (50 mM potassium phosphate, 1 mM PMSF, and 2 mM EDTA at pH 8). A fair amount of lipid-like substance was found in the fish liver during the enzyme extraction and removed by filter papers (Whatman, No.1). The suspension was centrifuged at 20,000*g* for 1 h to remove precipitates.

Step 2: 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 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 buffer B (10 mM potassium phosphate, 1.5 M ammonium sulfate, 1 mM PMSF, and 2 mM EDTA at pH 7). Then it was stirred gently for 120 min. Insoluble substances were removed by centrifugation (20,000*g*, 30 min). The dissolution of the 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. The 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 dialysis in 4 L of buffer C (10 mM potassium phosphate and 1 mM PMSF at pH 8) for 4 h with two changes of fresh buffer.

Step 4: chelating Sephacel. The enzyme solution in buffer C was applied to a column (1.6 × 10 cm) of chelating Sephacel (fast flow) that had been treated with 1 gel volume of 0.2 M zinc acetate. The column was then equilibrated with buffer D (20 mM potassium phosphate, 1 mM PMSF, and 0.5 M NaCl at pH 7). The loaded column was washed with 70 ml of 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). The active fractions were pooled and desalted by dialysis for 6 h with two changes of fresh 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). The enzyme was eluted by buffer C (total volume 500 ml) without a salt gradient. The pooled active fractions were applied to next step directly.

Step 6: hydroxyapatite Sephacel. The imidase solution was loaded into a column (1.6 × 10 cm) of hydroxyapatite Sephacel that had been equilibrated with buffer C at pH 7. Imidase was eluted with a linear potassium phosphate (pH 7) gradient from 0.02 to 0.4 M (total in a volume of 900 ml, including 1 mM PMSF). Homogeneous imidase

obtained from active fractions (70–85 fractions, 9 ml each) was pooled and then concentrated by ultrafiltration (YM 100 membrane), and frozen at −80 °C for later analysis. By the standard assay, the specific activity of fish imidase was 122 μmol/min/mg in a typical experiment. The specific activities of pig liver imidase and bacterial hydantoinase were 8 and 0.8 μmol/min/mg, respectively.

Results and discussion

Purification of cold-adapted imidase from fish liver

Imidase from fish liver was purified about 6000-fold with a 40% yield (Table 1). A homogeneous protein was obtained according to the criteria of SDS–PAGE (Table 1). The molecular weight of a single polypeptide chain was estimated to be around 56,000. The heat-treatment procedure was deliberately avoided during the purification so that the thermolabile fish imidase would not be deactivated. Previous investigations have confirmed that the heat treatment is quite useful in purifying other similar enzymes from calf [20] and pig [22] 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 [9]. However, the thermolabile imidase was not found in rat liver [9]. No thermolabile imidase has been reported previously.

Thermal stability and activity of fish liver imidase as compared to those of its thermophilic counterparts

Fig. 1 shows the difference in the thermostability of fish liver imidase and its thermophilic counterparts, pig liver imidase, and bacterial hydantoinase. The residual activity of fish liver imidase was significantly decreased in a few minutes followed by incubation at 50 °C. The stability was also affected by the variation of pH and resulted in the order of pH 8 > pH 7 > pH 6 > pH 9, for all three enzymes (data not shown). The half-lives (at 40 °C) of fish liver imidase in pH 6, 7, 8, and 9 were 50, 80, 150, and 40 min, respectively. The similar pH effects were previously observed for the imidase from pig liver

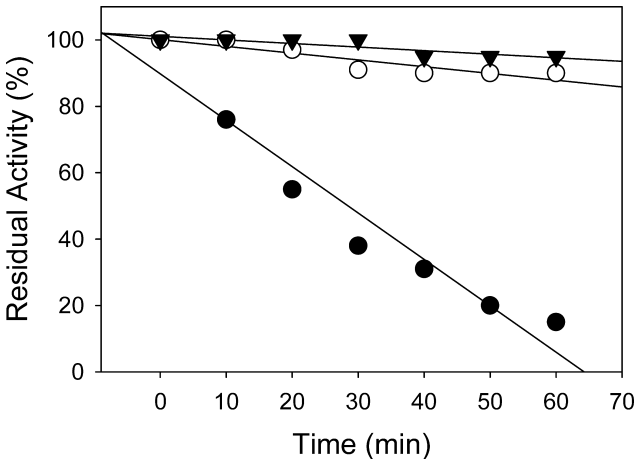


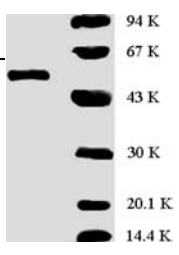
Fig. 1. Thermostability of imidase. Imidase (about 50 μg each in a final volume of 0.1 ml) from pig liver (○), fish liver (●), or bacteria (▼) was incubated at 50 °C in a Tris–HCl buffer (100 mM at pH 7.9). Aliquot amount of the incubated solution was removed at desired time and the imidase activity was determined by the standard assay. Each data point was the average of at least three measurements, which differed by less than 10%.

[11] and mesophilic *Bacillus sp.* AR9 [28], but they all have much longer half life spans (stable at 40 °C for 2 days or longer without loss of activity) than that of the fish liver imidase at elevated temperatures.

Fig. 2 shows the effects of temperature on the specific activity of the imidases. The specific activity of the fish liver imidase was much higher than that of the pig liver imidase except at 65 °C, due to the instability of fish liver imidase at elevated temperatures (Fig. 1). At temperatures lower than 50 °C, the specific activity of fish liver imidase was more than one order higher than that of pig liver imidase and two orders of magnitude higher than that of bacterial hydantoinase, according to the standard assay. The overall temperature effects on the activity among the three different sources of imidase were quite different. For example, the specific activity ratios (60/25 °C) were around 1.2, 5, and 3 for fish liver imidase, pig liver imidase, and bacterial hydantoinase, respectively (Fig. 2). Interestingly, the pig liver imidase and bacterial hydantoinase were nearly inactive in close proximity of

Table 1
Summary of purification of imidase from fish liver[a]

Step	Volume (ml)	Total activity (μmol/min)	Total protein (mg)	Specific activity (μmol/min/mg)	Yield (%)	Fold of purification
Extract	500	929	48,000	0.02	100	1.0
35% salt out	486	1290	16,524	0.08	139	4.1
60% salt out	75	1128	5175	0.22	121	11.6
Octyl Sepharose	198	832	970	0.86	90	45.3
Chelating Sephacel	70	619	36.4	17	67	895
DEAE Sephacel	30	597	8.6	70	64	3684
Hydroxyapatite	7	375	3.1	122	40	6421



[a] SDS–PAGE (12%) of purified fish liver imidase (56 kDa) and protein standards (from Pharmacia) with size noted in kilodaltons: phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), α-lactalbumin (14.4 kDa).

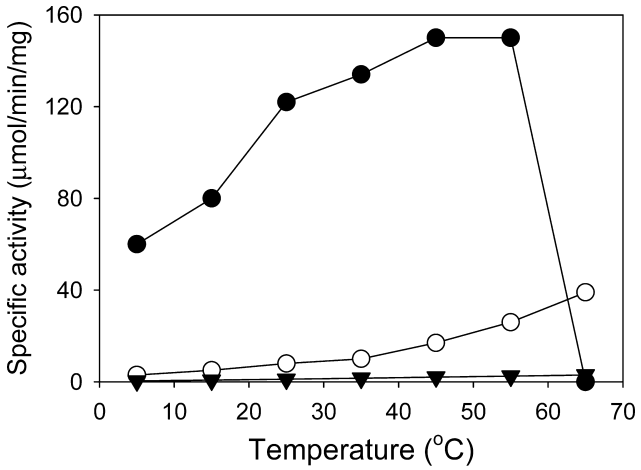


Fig. 2. Effect of temperature on the activity of imidase. The reaction mixture in the standard assay condition was pre-equilibrated at the desired temperature. The pH of the buffer (0.1 M Bis–Tris propane at pH 7) was determined at 25 °C and phthalimide was used as the substrate. The aliquot amount of imidase from pig liver (○), fish liver (●), or bacteria (▼) was added to start the reaction. Each data point was the average of at least three measurements, which differed by less than 10%.

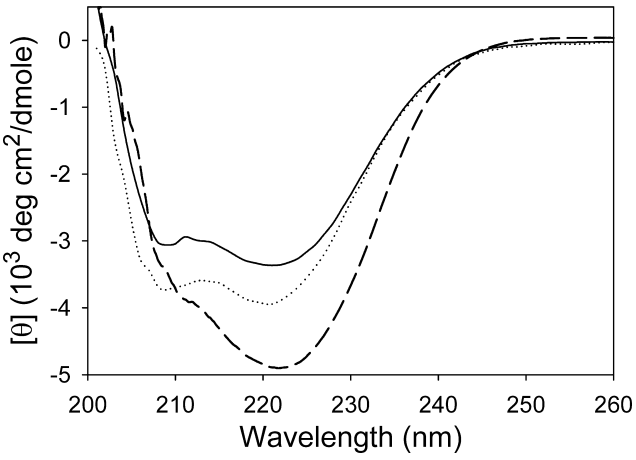


Fig. 3. Circular dichroism spectra of imidase. The circular dichroism spectra describe the imidases from pig liver (dotted line), fish liver (solid line), or bacteria (dashed line). The spectra were taken at room temperature by the Jasco Spectropolarimeter J-715. The enzyme (about 0.3 mg in 3 ml) was placed in a 10 mM potassium phosphate buffer at pH 7.

freezing point while the fish liver imidase was still quite active at this temperature. Since fish imidase plays a role in pyrimidine catabolism, it must work under freezing temperatures. The catalytic efficiencies (k_{cat}/K_m) of the three representative enzymes at 25 °C are listed in Table 2. It appeared that k_{cat} of imidase was mainly responsible for the dramatically different enzyme activities among the three enzymes compared. The imidase activities were substrate dependent as compared to the novel maleimide substrate (Table 2). Maleimide, which contains a conjugated imide, was first reported as the substrate of imidase and will be discussed further later.

Analysis of the secondary structure of imidases by circular dichroism

The circular dichroism spectra in the far-UV region of fish liver imidase, pig liver imidase, and bacterial hydantoinase at room temperature are shown in Fig. 3. Fish imidase gave the weakest signal in the far-UV region, which corresponded to an apparent lower fraction of secondary structures, as compared to those of pig liver imidase and bacterial hydantoinase. About 20%, 26%, and 40% α-helix contents for fish, pig liver, and bacterial hydantoinase, respectively, were estimated from the CD spectra using Yang's method [34]. Bacterial hydantoinase contains 29–31% α-helix according to their crystal structures [24,25]. It appeared that in the solution, fish liver imidase possessed a weaker optical activity and reflected less organized secondary structures. The thermostability (Fig. 1) of imidase appeared to be in a good correlation with its activity (Fig. 2) and secondary structure (Fig. 3) as would be expected.

Thermostability is believed to correlate with the amino acid composition, disulfide bridges, hydrophobic interaction, aromatic interaction, hydrogen bonds, and ion pairs of protein [35]. The high specific activity of fish liver imidase (Fig. 2) might relate to its possibly higher flexibility (Fig. 3) that might also lead to its instability (Fig. 1). Support of this postulation comes from the study of enzymes from thermophilic organisms that display high stability but low specific activity at ordinary temperatures [36]. The activation of a cold-adapted

Table 2
Substrate specificity of imidases[a]

Substrate	Imidase from	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m ($mM^{-1}\,min^{-1}$)	Specificity ratio (%)
Phthalimide	Fish	1.6 ± 0.2	25,700 ± 2800	16,100	100
	Pig	1.0 ± 0.07	1510 ± 60	1510	9
	Bacteria	5.8 ± 1.9	460 ± 120	79	0.5
Maleimide	Fish	200 ± 90	237,000 ± 109,000	1170	100
	Pig	13 ± 3	6400 ± 450	490	41
	Bacteria	180 ± 30	9800 ± 1200	54	5

[a] Enzymatic activity was measured in the standard assay condition except that different substrates and buffers (100 mM Tris–HCl at pH 7.9) were used at 25 °C. The reaction mixture was pre-equilibrated at 25 °C prior to the beginning of the enzymatic reaction. The pH of the solution was determined at 25 °C. K_m and k_{cat} were obtained by nonlinear regression (Enzyme Kinetic module of SigmaPlot). The extinction coefficient of each substrate was determined experimentally by direct measurement with a spectrophotometer.

enzyme may be explained by the lower global hydrophobicity of the enzyme [37]. The thermodynamic parameters of activation were used to explain the local flexibility of the cold-enzyme [38]. Despite the lack of specific answers for explaining cold-activity, there are some general models [39]. At lower temperatures the free energy barriers to these kinetic motions become greater and motion and breathing become restricted, which possibly interfere with catalysis. It is believed that cold-enzymes would be more flexible than those of their mesophilic counterparts and thus overcome this restriction [40]. Thus, in order for cold-adapted imidase to maintain high catalytic efficiencies (Fig. 2), there must be structural changes to enhance its activity (Fig. 3).

Maleimide was a novel substrate of imidase

Maleimide, a five-member ring imide containing conjugated double bonds, was first identified as a substrate of imidase and confirmed by HPLC and NMR analysis (Figs. 4 and 5). A more polar compound was produced following the incubation of fish liver imidase and maleimide as shown in Fig. 4B. Maleimide was stable in the absence of imidase at the incubation condition (Fig. 4A). Extra chemical shifts were produced following enzymatic reaction with maleimide as shown in Fig. 5B. These chemical shifts were identical to those of *cis*-3-amido-2-propenoic acid, the corresponding product of the hydrolysis of maleimide. Maleimide was also first reported here as a substrate for pig liver imidase and bacterial hydantoinase (Table 2). This observation was consistent with a previously proposed mechanism for

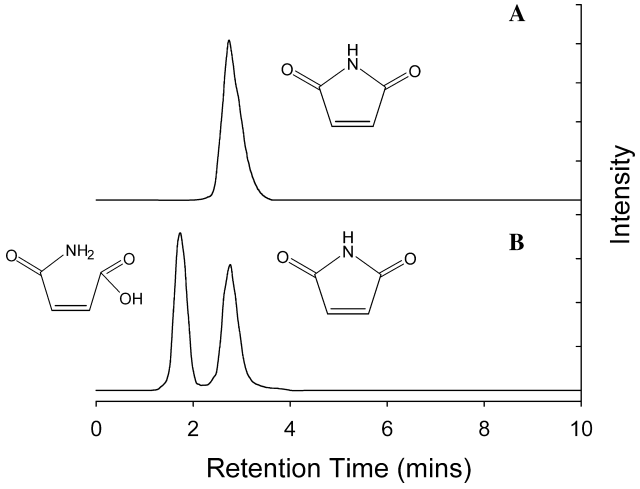


Fig. 4. HPLC analysis of maleimide before and after enzymatic hydrolysis. Maleimide and its hydrolyzing product were separated through a 5 μm (250 mm) pre-packed LiChrospher 100 RP-18 column (Merck, USA) and monitored at 339 nm with a UV–Vis detector using a D-7000 HPLC system (Hitachi, Japan). The separation was achieved in an isocratic eluent at a flow-rate of 1 ml/min. The HPLC mobile phase included 50% water and 50% acetonitrile. (A) Elution profile of 10 mM maleimide in phosphate buffer (10 mM, pH 6.5). (B) Elution profile of (A) after being incubated with 20 μg imidase for 2 h.

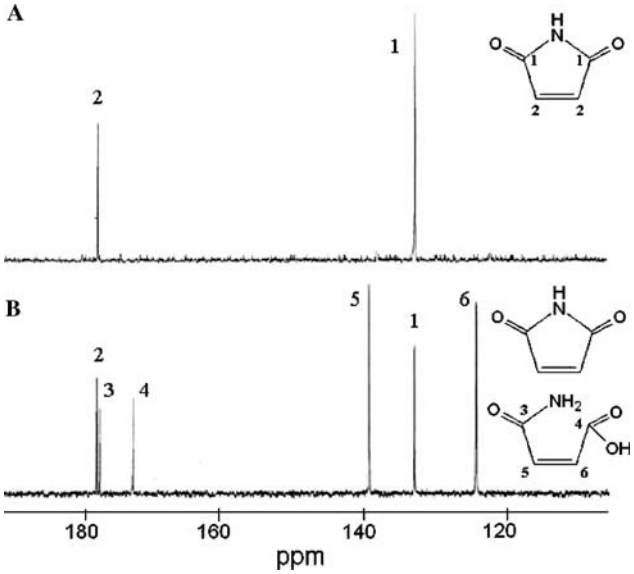


Fig. 5. NMR spectra of maleimide before and after enzymatic hydrolysis. The ^{13}C NMR spectra were recorded on a VARIAN UNITYINOVA 500 NMR spectrometer (VARIAN, USA) operating at a frequency of 125.7 MHz. Samples were placed in 5 mm NMR tubes and the spectra were recorded at room temperature. A pulse of 4.6 μs was used with an acquisition time of 0.508 s. The spectral width was set to 36.53 kHz and 37 kDa data points were recorded for every free induction decay. (A) The ^{13}C NMR spectrum 10 mM maleimide in phosphate buffer (10 mM, pH 6.5). (B) The ^{13}C NMR spectrum of (A) after being incubated with 20 μg fish imidase for 2 h.

imidase catalyzed imide hydrolysis [9]. A more planar structure was proposed [9] as an intermediate step through imide hydrolysis according to the pH profiles of different imides [9] and crystal structures of dihydrouracils [41]. It was suggested that substrates with more planar structures were more suitable for imidase [9].

Conclusion

A novel enzyme from fish liver was purified and identified as cold-adapted imidase. A novel imidase substrate, maleimide, was identified for all three enzymes studied. The specific activity, thermostability, and thermoactivity of fish liver imidase exhibited distinct differences with those of its thermophilic counterparts.

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