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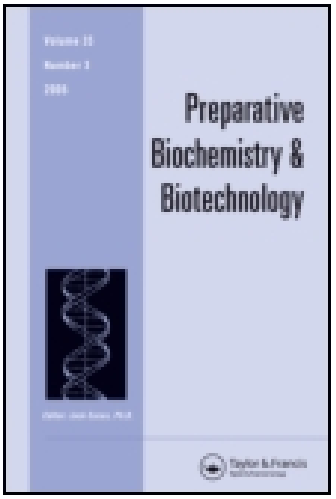

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Partial Purification and Characterization of A Novel Histidine Decarboxylase from Enterobacter aerogenes DL-1

Yu Zou [a], Wenzhong Hu [a], Aili Jiang [a] & Mixia Tian [a]

[a] College of Life Science, Dalian Nationalities University, Dalian, China

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Partial Purification and Characterization of A Novel Histidine Decarboxylase from *Enterobacter aerogenes* DL-1

Yu Zou, Wenzhong Hu, Aili Jiang, and Mixia Tian

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Histidine decarboxylase (HDC) from *Enterobacter aerogenes* DL-1 was purified in a three-step procedure involving ammonium sulfate precipitation, Sephadex G-100, and DEAE-Sepharose column chromatography. The partially purified enzyme showed a single protein band of 52.4 kD on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The optimum pH for HDC activity was 6.5, and the enzyme was stable between pH 4 and 8. *Enterobacter aerogenes* HDC had optimal activity at 40°C and retained most of its activity between 4 and 50°C. HDC activity was reduced in the presence of numerous tested compounds. Particularly with SDS, it significantly ($p < 0.01$) inhibited enzyme activity. Conversely, Ca^{2+} and Mn^{2+} showed prominent activation effects ($p < 0.01$) with activity increasing to 117.20% and 123.42%, respectively. The Lineweaver–Burk plot showed that K_m and V_{max} values of the enzyme for L-histidine were 0.21 mM and 71.39 μmol/min, respectively. In comparison with most HDCs from other microorganisms and animals, HDC from *E. aerogenes* DL-1 displayed higher affinity and greater reaction velocity toward L-histidine.

Keywords *characterization,* Enterobacter aerogenes*, histidine decarboxylase, purification*

INTRODUCTION

The presence of histamine in food is of concern to researchers, consumers, food companies, and health authorities due to its toxicological effects. Histamine at high concentration can cause histamine poisoning, which typically lasts up to 24 hr, producing allergy-like symptoms such as facial flushing, nausea, and headache.[1,2] The accumulation of histamine in food is a result of bacterial histidine decarboxylase (HDC, EC 4.1.1.22), which catalyzes the decarboxylation of L-histidine to form histamine.[3] These foods include fisheries products, cheese and wine, and other fermented products.[4] Thus, research on biochemical characterizations of HDC is helpful to fully understand the mechanism of histamine formation in food and avoid food poisoning caused by histamine.

Many bacterial species are known to possess histidine decarboxylase and have the ability to produce histamine. Most of them have been identified as the enteric bacteria that include *Proteus vulgaris, Proteus mirabilis, Enterobacter aerogenes, Enterobacter cloacae, Serratia fonticola, Serratia liquefaciens*, and *Citrobacter freundii*.[2,5] Among these enteric bacteria,

Address correspondence to Yu Zou, College of Life Science, Dalian Nationalities University, 18 Liaohe West Road, 116600 Dalian, China. E-mail: yuzou126@126.com

E. aerogenes is considered to be the strongest histamine-producing bacterium.[6,7] For a tuna dumpling stuffing contaminated with *E. aerogenes* stored at 25°C for 12 hr, histamine formed will be above 50 mg/100 g of the potential hazard level in most illness cases.[8] More accumulation of histamine is associated with higher HDC activity. Therefore, *E. aerogenes* also is considered to be one of the main HDC producers.

Many HDCs have been characterized in detail from various sources, such as *Tetragenococcus muriaticus*[4] among the gram-positive bacteria, *Morganella morganii*[9] among the gram-negative bacteria, and rat fetal liver[10] among the mammals. Some characterizations of HDC from *E. aerogenes* ATCC 43175 have been described in previous literature.[11] However, there is not other further information available in the literature about the characterization of *E. aerogenes* HDC isolated from marine fish. For the present study, a novel HDC produced by *E. aerogenes* DL-1 isolated from chub mackerel (*Scomber japonicus*) was purified and its partial biochemical characterizations were investigated and discussed.

MATERIALS AND METHODS

Microorganism and Maintenance

The bacterial strain *E. aerogenes* DL-1 was kindly provided by the Food-borne Pathogenic Microorganisms Fast Detection and Control Engineering Technology Research Center (Liaoning Province, China), and this was a strong histamine-producing bacterium previously isolated from chub mackerel (*Scomber japonicus*). Stock cultures were maintained on trypticase soy agar slants and subcultured every month.

Cultural Conditions for HDC Production

For production of the inoculum, the bacterial isolates were inoculated onto 50 mL seed medium (trypticase soy broth medium) in an Erlenmeyer flask (250 mL) and then incubated at 37°C for 24 hr with shaking at 150 rpm. The inoculum (5%) was transferred into an Erlenmeyer flask (250 mL) containing 50 mL of fermentation medium (trypticase soy broth supplemented with 1% L-histidine) and then cultivated at 37°C for 24 hr at a rotation speed of 150 rpm.

HDC Activity Assay

Assessment of HDC activity was performed by the method previously described by Shore et al.[12] A proper volume of the enzyme was added to the reaction solution containing 10 m*M* histidine (used as the substrate), 1 mg/mL bovine serum albumin, and 0.1 *M* phosphate buffer (pH 6.8), and the reaction was allowed to proceed for 15 min at 37°C. The reaction was stopped by adding 4 *M* perchloric acid, followed by centrifugation at 4,000 rpm for 5 min, and the resulting reaction mixture was extracted with *n*-butanol containing 5 *M* NaOH and 5 *M* NaCl. After neutralization, 1 mL of the resulting reaction mixture was added to the detection solution containing 1 *M* NaOH and *o*-phthalaldehyde and the relative fluorescence was measured by a fluorescence spectrometer (RF-5301, Shimadzu Co., Kyoto, Japan) at an excitation wavelength of 360 nm and emission wavelength of 450 nm.

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Protein Concentration Determination

Protein concentration was measured by the method of Bradford[13] using bovine serum albumin as the standard. In column chromatography elution, the amount of protein was measured in terms of the absorbance at 280 nm. The specific activity was expressed as the enzyme activity per milligram of protein.

Purification of HDC

Bacterial cells were collected from the culture by centrifugation at 8,000 rpm for 20 min at 4°C, washed twice with deionized water, and then resuspended in 0.1 *M* phosphate buffer (pH 6.8). The cells were disrupted with an ultrasonic disintegrator (JT-100, Ningbo Scientz Biotechnology Co., Ltd., Ningbo, China) at 250 W for 20 min at 4°C and the cell-free extract was obtained by centrifugation (10,000 rpm, 20 min). The enzyme in the cell-free extract was precipitated with 80% ammonium sulfate at 4°C. The precipitated enzyme was dissolved in a small amount of 0.1 *M* phosphate buffer (pH 6.8), and dialyzed overnight against the same buffer. The dialysate was loaded onto a Sephadex G-100 column (1.6 cm × 64 cm) equilibrated with 0.1 *M* phosphate buffer (pH 6.8). After washing the column with the same buffer, the proteins were fractionated and analyzed for enzyme activity. Active fractions were combined and concentrated with an ultrafiltration device (Amicon 8400, Millipore Corp., Billerica, MA) with a molecular mass cutoff of 10 kD. The concentrated enzyme solution was fractionated by anion-exchange chromatography on a DEAE-Sepharose column (1.6 cm × 64 cm) also equilibrated with 0.1 *M* phosphate buffer (pH 6.8). The enzyme was eluted using 0.1 *M* phosphate buffer (pH 6.8, containing different concentrations of sodium chloride). The active fractions were pooled and used for enzyme characterization.

Electrophoresis Analysis

The subunit molecular mass of HDC was estimated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli.[14] Gels containing 3% (stacking gel) and 12% (separation gel) acrylamide were prepared from a stock solution of 30% by weight of acrylamide and 0.8% by weight of *N,N′*-bis-methylene acrylamide. Ten-centimeter gels were prepared in glass tubes of a total length of 15 cm and with an inside diameter of 6 mm. The electrode buffer (pH 8.3) contained 0.025 *M* Tris, 0.192 *M* glycine and 0.1% SDS. The voltages used for stacking and separation of the proteins were 150 V and 300 V, respectively. Molecular mass markers in the range of 14.4–94.0 kD were used. To visualize protein, gel was stained with 0.1% solution of Coomassie brilliant blue R-250.

Effects of Ph and Temperature on HDC Activity and Stability

The effects of pH on enzyme activity were investigated with different pH ranging from 4 to 10 at 37°C for 30 min. The highest enzyme activity (approximately 10 U/mL) was used as control (100% of relative activity). The pH of reaction mixture was adjusted using 0.1 *M* on one of

the following buffers: sodium acetate-HCl buffer (pH 4–5), phosphate buffer (pH 6–8), and Tris-HCl buffer (pH 9–10). For pH stability, the enzyme was preincubated in the pH range of 4 to 10 at 4°C for 5 days in the absence of substrate. The optimal temperature for the enzyme activity was determined at temperature from 10°C to 80°C in 0.1 *M* phosphate buffer (pH 6.8) for 30 min. To determine the thermostability, the enzyme was incubated at various temperatures (4–80°C) in 0.1 *M* phosphate buffer (pH 6.8) for 60 min, and the relative residual enzyme activity was determined as described earlier.

Effects of Various Compounds on Enzyme Activity

The effects of various compounds on enzyme activity were determined by adding different metal ions and other reagents to the reaction mixture, preincubating the mixture at 4°C for 60 min, and then measuring the enzyme activity. The relative activity (approximately 10 U/mL) assayed in the absence of metal ion or reagent was recorded as 100%.

Kinetic Parameters of HDC

The reaction rates of HDC (approximately 20 U/mL) with substrate L-histidine was measured at various concentrations. The resulting data were analyzed and the Michaelis–Menten constant (K_m) and the maximal reaction velocity (V_{max}) of HDC were determined by Lineweaver–Burk plots.[15]

Statistical Analysis

The experimental results obtained were expressed as means ±SD of triplicates. Statistical analysis was performed using Fisher's *F*-test; $p < 0.05$ was regarded as significant and $p < 0.01$ as very significant.

RESULTS AND DISCUSSION

Purification of HDC

The results of HDC purification in each step are summarized in Table 1. Proteins were precipitated when the crude enzyme was saturated to 80% with ammonium sulfate. Approximately 90.32% of the enzyme yield was obtained with 1.33-fold purification. The partially purified enzyme was further purified by Sephadex G-100 column chromatography. Five peaks containing protein were observed and the fourth peak showed HDC activity (Figure 1). The enzyme was purified 4.27-fold with a yield of 77.34%. Finally, the active fraction was purified using anion-exchange column chromatography to separate HDC from most of other proteins. Six peaks containing protein were eluted from DEAE-Sepharose column and the fifth peak showed HDC activity (Figure 2). The 18.29-fold purification was obtained after anion-exchange chromatography with a yield of 36.49% and a specific activity of 1186.85 U/mg.

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TABLE 1
Purification of HDC From *E. aerogenes* DL-1

Purification step	Volume (mL)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude enzyme	1000.00	289.69	18800	64.90	100.00	1.00
Ammonium sulfate (80%)	150.00	197.28	16980	86.07	90.32	1.33
Sephadex G-100	21.00	52.41	14540	277.42	77.34	4.27
DEAE-Sepharose	35.00	5.78	6860	1186.85	36.49	18.29

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The purified HDC from *E. aerogenes* DL-1 showed a single protein band on SDS-PAGE and migrated with a molecular mass of approximately 52.4 kD (Figure 3). The different subunit molecular masses of HDCs were obtained from other organisms, such as 13 and 29 kD from *T. muriaticus*,[4] 11 and 28 kD from *Oenococcus oeni*,[16] 9 and 25 kD from *Lactobacillus buchneri*,[17] 10.5 and 25 kD from *Clostridium perfringens*,[17] 9 and 25 kD from *Lactobacillus* sp.,[18] 8 and 29 kD from *Micrococcus* sp.,[19,20] 9 and 26 kD from *Staphylococcus epidermidis*,[21] 43 kD from *Morganella morganii*,[9] 42.5 kD from *Klebsiella planticola*,[11] 50 kD from *E. aerogenes* ATCC 43175,[11] and 54 kD from rat fetal liver.[10] It appears that HDCs of different species may have different subunit molecular masses.

Effects of Ph on HDC Activity and Stability

The effects of pH on enzyme activity and stability are shown in Figure 4. Enzyme activity increased with the increase of pH, but it gradually decreased when pH was above 6.5. Therefore, the optimum pH for HDC activity was 6.5. The enzyme activity was stable over a broad pH range (pH 4–8) when incubated 5 days at 4°C. However, only 58.07% activity was retained at pH 9.2, which indicated that HDC was unstable in alkaline aqueous solution.

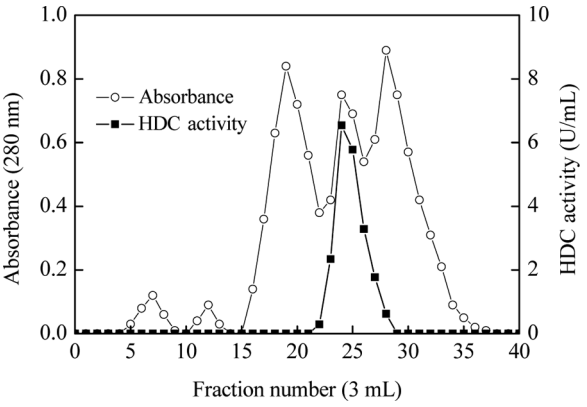

FIGURE 1 Profile of Sephadex G-100 chromatography of HDC from *E. aerogenes* DL-1. The enzyme was eluted using 0.1 *M* phosphate buffer (pH 6.8).

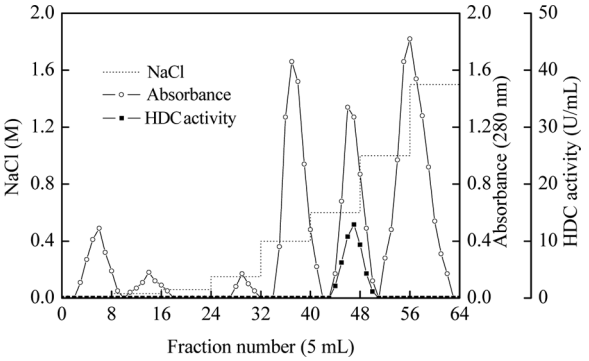

FIGURE 2 Profile of DEAE-Sepharose chromatography of HDC from *E. aerogenes* DL-1. The enzyme was eluted using 0.1 *M* phosphate buffer (pH 6.8, containing different concentrations of sodium chloride).

The purified HDC from *E. aerogenes* DL-1 exhibited optimum enzyme activity at pH 6.5 with a fairly wide range of pH stability from 4 to 8. Optimal values of other HDCs had been reported as pH 4.5–7.0 for *T. muriaticus*,[4] pH 4.8 for *O. oeni*,[16] pH 5.5 for *L. buchneri*,[17] pH 4.5 for *C. perfringens*,[17] pH 4.8 for *Lactobacillus* sp.,[18] pH 4.4–5.8 for *Micrococcus* sp.,[19,20] pH 6.0 for *S. epidermidis*,[21] pH 6.5 for *M. morganii*,[9] pH 6.5 for *K. planticola*,[11] pH 6.5 for *E. aerogenes* ATCC 43175,[11] pH 7.2–8.0 for rat and rabbit brain,[22] and pH 6.8 for rat fetal liver.[10] Compared with HDCs from gram-positive bacteria and animals, the optimum pH of HDC from *E. aerogenes* DL-1 was more close to those of HDCs from gram-negative

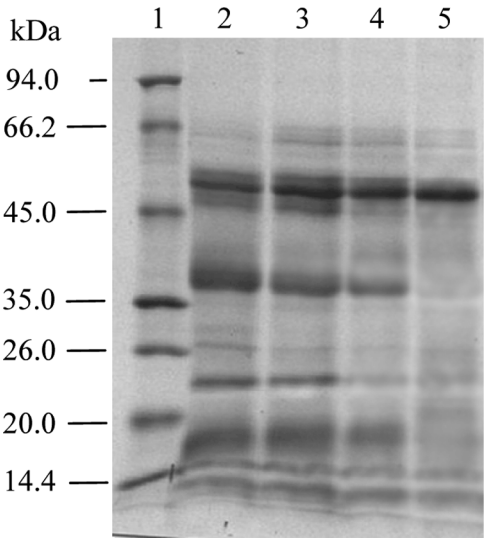

FIGURE 3 SDS-PAGE analysis of purified HDC from *E. aerogenes* DL-1. Lane 1: molecular mass marker proteins (Pharmacia, USA); Lane 2: crude enzyme; Lane 3: ammonium sulfate precipitation; Lane 4: Sephadex G-100 filtration; Lane 5: DEAE-Sephcrose purification.

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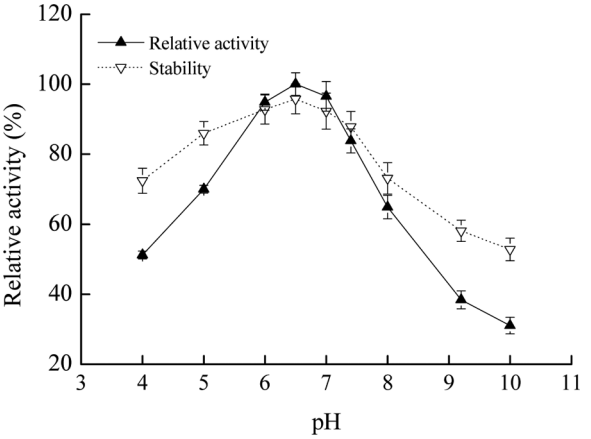

FIGURE 4 Effects of pH on the relative activity and stability of HDC from *E. aerogenes* DL-1. The enzyme activity was assayed by the standard assay method by changing the buffer to obtain the desired pH. The buffers used were sodium acetate-HCl (pH 4–5), phosphate (pH 6–8), and Tris-HCl (pH 9–10). Values are represented as the means ± SD ($n = 3$).

bacteria. Some other investigators had also reported that HDCs were stable in the range of pH 4–7,[4,18,21] which agreed with the data obtained in this study.

Effects of Temperature on HDC Activity and Stability

The effects of temperature on enzyme activity and stability are presented in Figure 5. There was a gradual increase in enzyme activity with increasing temperature, but a sharp decrease occurred when temperature exceeded 40°C. Thus, the optimum temperature for HDC activity was 40°C.

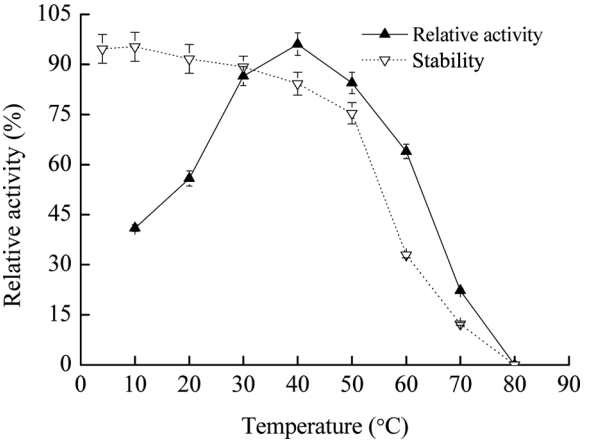

FIGURE 5 Effects of temperature on the relative activity and stability of HDC from *E. aerogenes* DL-1. The enzyme activity was assayed at various temperatures by the standard assay method. Values are represented as the means ± SD ($n = 3$).

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According to the thermostability assay, HDC was highly stable up to 50°C. At temperatures over 50°C, the thermostability of HDC sharply decreased and the enzyme activity was completely inactivated at 80°C.

An optimal temperature is very important for the reaction of an enzyme. At lower temperature the enzyme is not activated, while high temperature may destroy the spatial structure of the enzyme and result in the loss of catalytic ability. As was shown in Figure 5, the purified HDC from *E. aerogenes* DL-1 showed maximum activity at 40°C. This value was lower than optimum temperature of *Staphylococcus epidermidis* HDC, which was reported to have an optimum temperature of 60°C.[21] However, optimum temperature of HDC from *E. aerogenes* DL-1 was in accordance with most previous reports indicating that the optimum temperature of 40–45°C was obtained from *T. muriaticus*,[4] *M. morganii,*[9] and rat and rabbit brain.[22] HDC from *E. aerogenes* DL-1 was stable below 50°C, but above this temperature the thermostability decreased sharply. This result was similar to those of HDCs reported by Konagaya et al.[4] and Snell,[23] indicating that HDCs from other sources also were significantly unstable at a temperature of 60°C or higher.

Effects of Various Compounds on HDC Activity

The effects of potential inhibitors or activators on the purified enzyme are shown in Table 2. The result displayed that numerous tested compounds had an inhibitory effect on enzyme activity. Cu^{2+}, Ba^{2+}, Tween-80, Triton X-100, and ethylenediamine tetraacetic acid (EDTA) significantly ($p < 0.05$) decreased HDC activity by 13.33, 12.55, 10.91, 12.47, and 7.79%, respectively. SDS, an anionic detergent, had the most prominent inhibitory effect ($p < 0.01$), with activity decreasing to 69.45%. Conversely, the enzyme activity was markedly ($p < 0.01$) activated by Ca^{2+} and Mn^{2+}. In particular for Mn^{2+}, it significantly enhanced enzyme activity approximately 1.23-fold. In addition, Na^{+}, K^{+}, Mg^{2+}, and Zn^{2+} caused practically no effect ($p > 0.05$) on enzyme activity in the tested concentration.

TABLE 2
Effects of Metal Ions and Chemical Reagents on HDC Activity

Chemicals	Concentration	Relative activity (%)
None		100 ± 0.0
Na^{+}	5 m*M*	102.29 ± 3.51 NS
K^{+}	5 m*M*	101.17 ± 3.32 NS
Mg^{2+}	5 m*M*	103.34 ± 4.12 NS
Ca^{2+}	5 m*M*	$117.20 \pm 4.51^{**}$
Cu^{2+}	5 m*M*	$86.67 \pm 3.41^{*}$
Zn^{2+}	5 m*M*	98.20 ± 2.77 NS
Mn^{2+}	5 m*M*	$123.42 \pm 5.88^{**}$
Ba^{2+}	5 m*M*	$87.45 \pm 2.46^{*}$
Tween-80	0.5%	$89.09 \pm 1.29^{*}$
Triton X-100	0.5%	$87.53 \pm 3.56^{*}$
SDS	0.5%	$69.45 \pm 2.94^{**}$
EDTA	0.5%	$92.21 \pm 3.85^{*}$

Note. Values are represented as the means ± SD ($n = 3$). Significance: $^{**}p < 0.01$; $^{*}p < 0.05$; *NS*, not significant.

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The effects of metal ions and chemical reagents on HDC activity were diverse (Table 2). In the tested compounds, SDS strongly ($p < 0.01$) inhibited the enzyme activity. This might be because SDS could influence interfacial area between substrate and enzyme. In addition, SDS might directly destroy the catalytic site of the enzyme, resulting in the reduction of enzyme activity.[24] The HDC activity also was significantly ($p < 0.05$) inhibited by Cu^{2+}. However, the mechanism of action of Cu^{2+} has not been reported. The presence of Ca^{2+} and Mn^{2+} markedly ($p < 0.01$) enhanced the HDC activity, which agreed with results reported by Savany and Cronenberger.[25] It was possible that these cations might be cofactors of HDC and influence enzyme activity by changing the spatial structure.[22] Therefore, Ca^{2+} and Mn^{2+} were the primary contributors to enhancement of HDC activity.

Kinetic Parameters of HDC

Kinetic studies were carried out with the purified HDC. L-Histidine was used as substrate and its tested concentrations were 0.1, 0.2, 0.3, 0.4, 0.5, and 0.6 m*M*. The experimental results are shown in Figure 6. The Lineweaver–Burk plot showed that K_m and V_{max} values of the enzyme for L-histidine, at pH 6.8 and 40°C, were 0.21 m*M* and 71.39 μmol/min, respectively.

The K_m value of HDC from *E. aerogenes* DL-1 towards L-histidine was 0.21 m*M*, which was lower than the values reported previously for *T. muriaticus* (0.74 m*M*),[4] *Oenococcus oeni* (0.33 m*M*),[16] *L. buchneri* (0.6 m*M*),[17] *Lactobacillus* sp. (0.4 m*M*), [18] *Micrococcus* sp. (0.8 m*M*),[19,20] *S. epidermidis* (1.1 m*M*),[21] *M. morganii* (1.3 m*M*),[9] *K. planticola* (2.4 m*M*),[11] *E. aerogenes* ATCC 43175 (2.2 m*M*),[11] and rat fetal liver (0.5 m*M*).[10] The measured V_{max} value for L-histidine (71.39 μmol/min) was similar to those of *L. buchneri* (69 μmol/min) and *M. morganii* (73 μmol/min)[9] but approximately 2–5 times higher than the values of HDCs from *T. muriaticus*,[4] *O. oeni*,[16] *C. perfringens*,[18] *Micrococcus* sp.,[19,20] and *S. epidermidis*.[21] Compared with most of HDCs from other microorganisms and animals, HDC from *E. aerogenes* DL-1 possessed greater affinity and higher reaction velocity toward L-histidine.

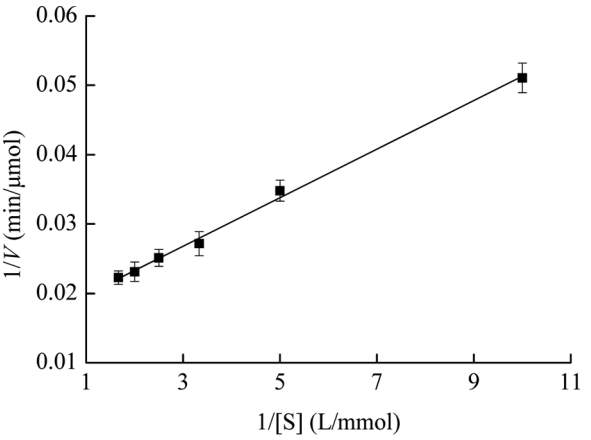

FIGURE 6 Lineweaver–Burk plot for K_m and V_{max} values of the purified HDC from *E. aerogenes* DL-1 in the presence of different concentrations of L-histidine. Each value represents the mean of triplicate measurements and variations from the mean are not more than 6%.

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CONCLUSIONS

A novel HDC produced by *E. aerogenes* DL-1, a strong histamine-producing bacterium isolated from chub mackerel (*Scomber japonicus*), was purified and its partial biochemical characterizations were investigated. The enzyme showed a high affinity to L-histidine and high catalytic properties. However, further work is required to delineate the behavior and characteristics of *E. aerogenes* HDC in actual fish products and to devise strategies for reducing histamine levels in these seafoods.

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