Purification and Characterization of a Hexanol-Degrading Enzyme Extracted from Apple

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ABSTRACT: An enzyme having activity toward n-hexanol was purified from apple, and its biochemical characteristics were analyzed. The purification steps consisted of sedimentation with ammonium sulfate, DEAE Sepharose Fast Flow ion exchange chromatography, and Sephadex G-100 column. The obtained enzyme had a yield of 16.00% with a specific activity of 18879.20 U/mg protein and overall purification of 142.77-fold. The enzyme showed activity to isoamylol, 1-propanol, n-hexanol, and isobutanol but not toward methanol and ethanol. With n-hexanol as a substrate, the optimum conditions were pH 4.0 and 30 °C for enzyme activity and pH 3.0–4.0 and temperatures below 40 °C for enzyme stability. The enzyme activity was increased significantly by adding L-cysteine and Fe^{2+} at all tested concentrations and slightly by Zn^{2+} at a high concentration but decreased by additions of EDTA, Ga^{2+}, K^+, Mg^{2+}, sodium dodecyl sulfate (SDS), sodium aluminum sulfate (SAS), dithiothreitol (DTT), and glutathione (GSH). The enzyme activities toward n-hexanol and n-hexanal were increased by NADH but decreased by NAD^+, in contrast to a decrease toward n-hexane by addition of both NAD^+ and NADH.

KEYWORDS: enzyme, apple, purification, characteristics, kinetic, higher alcohol, n-hexanol

INTRODUCTION

Alcohols with more than two carbon atoms are commonly called higher or fusel alcohols.[1] Higher alcohols are formed by yeast metabolism from amino acids and sugars and therefore are normal constituents naturally found in alcoholic beverages derived from alcohols of agricultural origin (Erlich mechanism).[2,3] They have an aromatic effect in wine, which could be positive. An excessive concentration of the higher alcohols could distort food flavor and cause damage to the human nervous system.[4] The higher alcohols in alcoholic beverages mainly include 1-propanol (*n*-propyl alcohol), 1-butanol (*n*-butyl alcohol), 2-butanol (*sec*-butyl alcohol), isobutanol (2-methyl-1-propanol), *n*-hexanol, and isoamyl alcohol (3-methyl-1-butanol).[1,5] The total higher alcohol content was reported as 162–266 mg/L for white wines and 140–417 mg/L for red wine produced in California.[6] The higher alcohols were oxidized slowly, resulting in a long time in the body and increased toxicity level.[7] Hexanol was also found in alcohol beverages in a lower level than the other higher alcohols but showed a higher toxicity potential to animals due to its lower oral LD_{50} value in rats of 720 mg/kg as compared to 1-butanol (790 mg/kg), isoamylol (1300 mg/kg), 1-propanol (1870 mg/kg), 2-butanol (2193 mg/kg), and isobutanol (2460 mg/kg).[1] A preliminary guideline level was set at 500 g/L of pure alcohol for the sum of all higher alcohols on the daily assumption of alcoholic drink and the maximum tolerable concentration of each higher alcohol.[1] This level is higher than the concentrations normally found in both legal alcoholic beverages and surrogate alcohols.[1] In China, every year, a hexanol content of up to 2000 mg/L has been found in 20% samples of alcoholic beverages, including wine and liquor. At present, hexanol has been listed as a Hazardous Substance that can cause harm to the human body (HSDB, Hazardous Substances Data Bank).

The yeasts, producers of higher alcohols at low contents, were explored for alcoholic fermentation to reduce these substances in final products.[5,8−10] However, the alcohol production was reduced, and flavor was also altered by using such yeasts due to the depression of alcohol metabolism in the strains. High ethanol-producing capability is important for yeasts to avoid the toxicity of secondary alcohols through competitive inhibition. Enzymatic treatment in vitro[11] and absorption by raisin[12,13] were also explored to reduce the higher alcohols in alcoholic drinks. The enzymatic treatment was supposed as a potential way to achieve the reduction in higher alcohols due to its high specificity and safety. Therefore, a high specificity toward higher alcohols without activity to ethanol is essential for the enzyme when the alcohol flavor is considered. Alcohol dehydrogenase was normally considered for the reduction of alcohols in food processing due to the oxidized products that tended to aldehyde and ester, which contribute to good flavors in subsequent reaction procedures. Up to now, little research has been focused on exploring enzymes that are specific to higher alcohols without activity to ethanol. Most of the enzymes that have activity to higher alcohols, especially *n*-hexanol, also have activity to ethanol.

Our research has been focused on developing a *n*-hexanol-degradating enzyme that is isolated from apple and shows high specificity and activity toward higher alcohols but not toward ethanol. To understand the biochemical characteristics of this enzyme, this study was aimed at studying the purification

procedures, substrate specificity, optimum pH, and temperature for activity and stability, metal ion effects, inhibitor, and cofactor effects with *n*-hexanol as the substrate.

■ MATERIALS AND METHODS

Apple and Chemicals. Fresh and good quality apples (*Malus domestica* Borkh. CV. Red Fuji) at commercial maturity were obtained from a local store in Yangling City, Shaanxi Province, China, in November, 2010. All chemicals used in the study were of analytical grade. *n*-hexanol, methanol, ethanol, isoamylol, 1-propanol, and isobutanol were purchased from Sinopharm Chemical Reagent Co.Ltd. (Ningbo, China). Nicotinamide adenine dinucleotide phosphate (NADP⁺), nicotinamide adenine dinucleotide (NAD⁺), NADH, and NADPH were bought from Amresco (Solon, United States). Polyethylene glycol (PEG) 6000 and MD25 dialysis tubing were bought from Sigma (St. Louis, MO).

Methods. *Enzyme Preparation.* The enzyme extraction from apples was carried out using the method of acetone.[14] A sample of 200 g of fresh apple was homogenized in 50 mL of cold acetone (−30 °C) by using a prechilled commercial blender through mixing for 2 min at the maximum speed of 200 r/min. The homogenized mixture was filtered through an analytical fiber filter paper, and the residue was mixed with 100 mL of cold acetone, mixed, and filtered again. Such a cold acetone treatment was repeatedly conducted for five times. Finally, the residue was obtained as the crude enzyme, a white powder. The crude enzyme powder was further dried overnight at room temperature and stored at −20 °C before use. The yield of crude enzyme from 200 g fresh apple was 5 g in dried powder.

To prepare the enzyme extract from the enzyme powder, the powder of 0.5 g was suspended in 37.5 mL of prechilled 0.1 M citrate buffer at pH 4.0 and stirred for 30 min at 4 °C. Then, the suspension was centrifuged at 4000*g* for 30 min in an ALC PK121R Refrigerated Benchtop Centrifuge (ALC International S.r.l., Cologno Honzese, Italy) at 4 °C. The supernatant was collected and used as an enzyme extract for purification.

Purification of Enzyme. Purification of the enzyme was carried out on the prepared enzyme extract following a three-step procedure described by Pang et al.[15]

Ammonium Sulfate Treatments. Samples were first precipitated with ammonium sulfate using the fractions at 30 and 70% concentrations, respectively, according to the preliminary experiments. The precipitates deposited by the two different fractions of ammonium sulfate were separately collected by centrifugation at 4 °C and 10000*g* for 10 min using a High Speed Refrigerated Centrifuge HTC-24SMTI (Abbota, NJ). The deposited pellets were suspended in a minimal volume of 0.1 M citrate buffer at pH 5.4.

DEAE-Sepharose Fast Flow. The enzymes from ammonium sulfate fractions were subjected to DEAE-Sepharose FF column (1.6 cm × 40 cm) equilibrated with 20 mM citrate buffer at pH 5.8. After the column was washed with the same buffer, bound proteins were eluted with a linear gradient of sodium chloride (0−1.2 M) in the equilibrating buffer. Fractions (5 mL each) were collected at a flow rate of 5 mL/5 min. The protein content and the enzyme activity toward *n*-hexanol of each fraction were measured using the methods described in the sections Protein Determination and Measurement of Enzyme Activity. The fractions showing activity were collected and used for the next step.

Sephadex G-100 Gel Filtration. The enzyme fractions with activity toward *n*-hexanol were applied to a gel filtration using a Sephadex G-100 column (1.8 cm × 100 cm) equilibrated with 20 mM citrate buffer at pH 5.8. The fractions of 5 mL were collected at a flow rate of 5 mL/20 min with the same buffer. After the enzyme activity of each fraction toward *n*-hexanol was measured, the fractions with activity toward hexanol were pooled and concentrated to 4 mL by using dialysis tubing of 8−1.4 kDa cutoff embedded in crystalline PEG 6000 (Sigma Chemical Co., United States) for 60 min at 4 °C. The concentrated enzyme was stored at −20 °C for further analysis of characteristics and stability.

Polyacrylamide Gel Electrophoresis. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to determine the purity and molecular mass of the enzyme as described by Laemmli[16] using a 4% (w/v) stacking gel and a 12% (w/v) separating gel. The molecular weight of the enzyme was estimated by using protein markers with known molecular weight: myosin (200 kDa), *β*-galactosidase (116 kDa), phosphorylase B (97.2 kDa), serum albumin (66.4 kDa), ovalbumin (44.3 kDa), carbonic anhydrase (29.0 kDa), trypsin inhibitor (20.1 kDa), lysozyme (14.3 kDa), and aprotinin (6.5 kDa). After SDS-PAGE, proteins on the polyacrylamide gel were stained with 0.2% Coomassie brilliant blue R-250.

Protein Determination. Protein concentrations were measured according to the method of Bradford, using bovine serum albumin (Bio-Rad) as the standard.[17]

Measurement of Enzyme Activity. The enzyme activity was determined by measuring the decrease of substrate. A sample of 300 µL of prepared enzyme (132 U/mg protein) was mixed with a 2.7 mL assay mixture (pH 4.0) containing 0.1 M citrate and 9.8 mM substrate and kept at 30 °C for 30 min, which is the period in the linear part of the reaction based on our preliminary experiments. Unless indicated, the substrate was *n*-hexanol. The pH for the activity measurement was set at pH 4.0 because apple and most of alcoholic beverages have pH values about 4.0. The decrease of substrate was followed for 30 min in a water bath shaker at 30 °C to determine the enzyme activity. Then, the ice bath (10 min) was used to stop the reaction, and the sample was stored at −40 °C before a gas chromatograph measurement was made. The mixture of solution with citrate buffer substituting the enzyme solution was treated at the same conditions and taken as the control in the measurement. The enzyme activity was expressed as the decrease of substrate concentration (mM) per minute at a certain quantity of the enzyme. One unit (U) of the purified enzyme is the amount of enzyme required to reduce 1 µmol of *n*-hexanol per min under the assay conditions. Determination of the decrease of higher alcohols was performed with gas chromatographic analysis.[18,19]

The GC analyses were performed using a headspace gas chromatograph (GC-17A/FID, Shimadzu, Japan) equipped with a flame onization detector (FID) and with a split−splitless injector, both set at 240 °C. Nitrogen was the carrier gas at a linear flow rate of 30 mL/min. Compounds were separated on a SupelcoWax 10 capillary column (30 m × 0.32 mm i.d., 0.25 µm film thickness), purchased from Bellefonte (PA). The column temperature was programmed as follows: 55 °C for 3 min, then at 15 °C/min up to 200 °C, with a final holding time of 3 min. Before determination of higher alcohols, the reaction mixture after different treatments was equilibrated at 60 °C in water bath for 30 min before the vapor phase (300 µL) was injected in the GC system.

The content of the substrate in the reaction system was tested using headspace solid-phase microextraction coupled with gas chromatography−mass spectrometry (HS-SPME/GCMS). Briefly, a Supelco divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMSCW/DVB) 50/30 µm fiber was exposed to the headspace of sample vials (8 mL) for 10 min under 30 °C water bath temperature and desorbed in the GC inlet for 5 min. A gas chromatograph was equipped with a mass spectrometer detector (Finnigan, United States). The chromatographic column was a capillary column DB-WAX (3 m × 0.25 mm i.d. × 0.25 µm film thickness, Agilent, United States), and the oven temperature program was 40 °C (2.5 min), while the SPME inlet linear was held at 250 °C, 5 °C/min up to 200, and 10 °C/min up to 240 °C (5 min). The flow rate of carrier gas (N₂) was 1 mL/min. The temperature of transfer line was 230 °C. Data acquisition was made in different segments with electronic impact mode. The range of masses was 35−350 *m/z*. For electron ionization (EI), the emission current was 100 µA. The identification of each compounds was carried out using the spectra obtained with standard compounds and from the NIST 2002 (Xcalibur).[20]

Substrate Specificity. Substrate specificity of the enzyme was investigated by using methanol, ethanol, 1-propanol, isobutanol, isoamylol, and *n*-hexanol as substrates in the mixture for enzyme activity described above. For each substrate, the enzyme activity was measured for a range of substrate concentrations from 10 to 50 mM.

The Michaelis−Menten constant (K_m), maximum rate of the reaction (V_{max}), and specificity (V_{max}/K_m) for the different substrates were determined by plotting the activity data as a function of substrate concentration according to the method of Lineweaver and Burk.[21]

Effect of pH and Temperature on Activity and Stability of Enzyme. The effects of temperature and pH on the enzyme activity and stability were examined by using *n*-hexanol as a substrate. To obtain the optimum pH for the enzyme activity, the enzyme activity was measured in the mixture of 0.1 M citrate buffer in a pH range of 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, and 8.0 at 30 °C for 30 min. The enzyme activities at different temperatures of 20, 30, 40, 50, 60, and 70 °C were also measured after incubation at pH 4.0 for 30 min to determine the optimum temperature. For investigating the enzyme stability under different pH and temperature levels, the enzyme was kept at different pH values from 2.0 to 8.0 at 30 °C for 24 h and at different temperatures ranging from 20 to 70 °C at pH 4.0 for 1 h before the enzyme activity was determined at 30 °C and pH 4.0. The keeping period was determined based on enzyme stability at different conditions through preliminary experiments, in which the enzyme activity was completely lost after 1.5 h at the temperature of higher than 50 °C and the difference among different pH levels was the most significant after 24 h in the pH test.

Effect of Metal Ions and Inhibitors on Enzyme Activity. With *n*-hexanol as a substrate, the effects of metal ion and inhibitors on the enzyme activity were investigated using $CaCl_2$, $FeSO_4$, KCl, $MgSO_4$, and $ZnSO_4$ as suppliers of metal ions of Ca^{2+}, Fe^{2+}, K^+, Mg^{2+}, and Zn^{2+} at different concentrations (0.01, 0.05, and 0.10 mM), chelator ethylenediaminetetraacetic acid (EDTA) at concentrations ranging from 0.2 to 1.0 mM, and surfactants SDS and sodium aluminum sulfate (SAS), denaturants dithiothreitol (DTT), and inhibitors of L-cysteine and glutathione (GSH) at different concentrations (0.05, 0.1, and 0.5 mM). Each metal ion was prepared in citrate buffer (pH 4.0). The investigation on the enzyme activity was carried out in a reaction mixture (3 mL) of 2.0 mL of *n*-hexanol (14.7 mM), 700 μL of metal ion solution or inhibitor solution, and 300 μL of enzyme extract. The reaction mixture was incubated for 30 min at 30 °C before the decrease of *n*-hexanol concentration was determined. The reaction mixture without enzyme was taken as blank to determine the decrease of *n*-hexanol for enzyme activity analysis. The enzyme activity obtained for the mixture without any extra ion or inhibitor was taken as the control, corresponding to 100% relative activity.

Cofactor Specificity. The effect of various cofactors on enzyme activity was investigated using NAD^+, $NADP^+$, NADH, and NADPH at 0.3 mM. The investigation on the enzyme activity was carried out in a reaction mixture (3 mL) of 2.0 mL of *n*-hexanol (14.7 mM), 700 μL of cofactor solution, and/or 300 μL of enzyme extract. The reaction mixture was incubated for 30 min at 30 °C before the decrease of *n*-hexanol concentration was determined. The activity of the enzyme in the absence of added cofactor was considered as 100%. The enzyme activities toward *n*-hexanol, *n*-hexane, and *n*-hexanal with final concentrations of 9.8, 11.6, and 9.98 mM, respectively, were also tested in presence of NAD^+ and NADH (0.3 mM) at 30 °C and pH 4.0.

Data Analysis. Office Excel 2007 was used to calculate the standard deviation of experimental data. The average of triplicates with standard deviation is reported in the paper.

■ RESULTS

Enzyme Purification. The enzyme was purified by a tristep procedure, and the results are presented in Figures 1 and 2 and Table 1. In the first step of ammonium sulfate precipitation, the enzyme was purified by 11.91-fold (Table 1). In the second step of column chromatography, fractions 21−31 with high enzymatic activities were eluted at a NaCl concentration of 0.3 M (Figure 1). The fractions had a protein concentration of 0.0079 mg/mL and a total enzymatic activity of 2583 U (Table 1). In the third step of ion-exchange column, the active fractions were loaded onto a Sephadex G-100 gel filtration. Fractions 5−22 showed the activity toward *n*-hexanol were
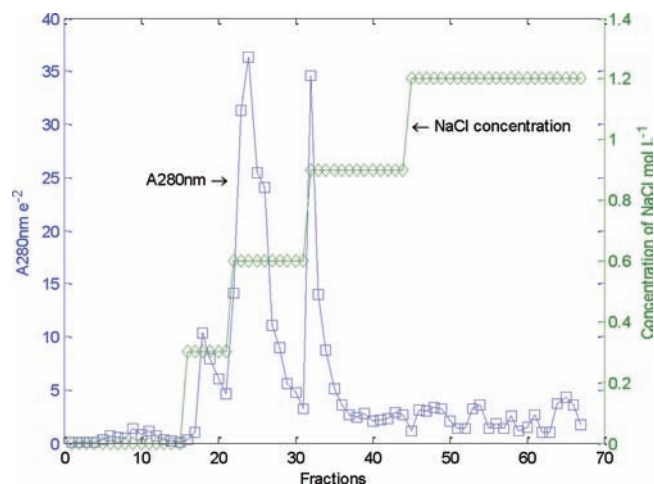

Figure 1. Ion-exchange column chromatography of the enzyme from apple fruit. The enzyme was applied to a DEAE-Sepharose column and eluted with a stepwise gradient ranging from 0 to 1.2 M NaCl in 20 mM citrate buffer (pH 5.8). Fractions 21−31 showed activity toward *n*-hexanol, with a protein concentration of 0.0079 mg/mL and a total activity of 2583 U.
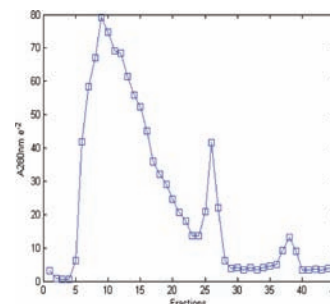

Figure 2. Gel-filtration column of the isolated enzyme. DEAE-Sepharose runoff fractions were pooled and then applied to a Sephadex G-100 column and eluted with 20 mM citrate buffer (pH 5.8). Fractions 5−23 showed activity toward *n*-hexanol.

collected using 20 mM citrate buffer at 4 °C (Figure 2). The resulting enzyme solution contained 0.0023 mg/mL protein, and the total enzymatic activity was 2475 U. Finally, the purification was 142.77-fold with 16% recovery of enzymatic activity toward *n*-hexanol. The SDS-PAGE result showed a single major protein band at molecular mass of 19.7 kDa (Figure 3).

Substrate Specificity and Enzyme Kinetics. Four different higher alcohols were used to test the substrate specificity, as well as methanol and ethanol. The enzyme showed activity toward higher alcohols of isobutanol, *n*-hexanol, 1-propanol, and isoamylol but not toward methanol and ethanol at all tested concentrations from 10 to 50 mM. Figure 4 shows that the enzyme kinetic followed a typical first order of Micheal function with *n*-hexanol, 1-propanol, and isoamylol as the substrates. Apparent K_m and V_{max} values for different substrates are listed in Table 2. 1-propanol showed the lowest K_m and V_{max} values as compared to other substrates. According to the value of V_{max}/K_m, the enzyme showed the highest affinity toward isobutanol followed in a decreasing order by *n*-hexanol, 1-propanol, and isoamylol. The high specificity to higher alcohols and no activity toward methanol and ethanol indicated the potential application of the enzyme in food processing where the higher alcohols are not desirable.

Table 1. Purification of the Enzyme from Apple Fruit[a]

purification steps	volume (mL)	total activity (U)	total protein (mg)	specific activity (U mg protein^{-1})	recovery (%)	purification fold
crude extract	500	15471.75	117.00	132.24	100.00	1
ammonium sulfate (30−70%)	96	7955.64	5.05	1575.50	51.42	11.91
DEAE-Sepharose FF	50	2583.53	0.40	6522.41	16.70	49.32
Sephadex G-100	57	2475.06	0.13	18879.20	16.00	142.77

[a]All experiments were conducted at 4 °C. The 30 and 70% ammonium sulfate fractions were pooled and then subjected to DEAE-Sepharose ion-exchange chromatography.
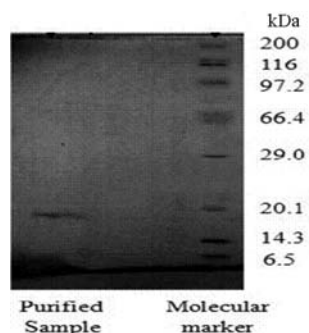

Figure 3. SDS-PAGE of the purified enzyme. The numbers on the right are the molecular masses of the markers.
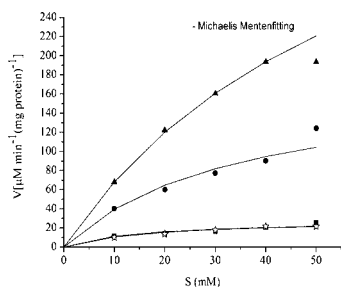

Figure 4. Enzyme activity toward different substrates as a function: isobutanol (closed triangle), *n*-hexanol (closed circle), 1-propanol (closed square), and 1-propanol (open star). The conditions used in measurement of the enzyme activity were pH 4.0 and 30 °C.

Table 2. K_m and V_{max} Values of the Enzyme toward Different Substrates[a]

substrate	K_m (mM)	V_{max} [μM min^{-1} (mg protein)$^{-1}$]	×10^{-3}V_{max}/K_m [min^{-1} (mg protein)$^{-1}$]
n-hexanol	34.21	175.44	5.13
isoamylol	16.23	28.55	1.74
isobutanol	63.20	434.78	7.91
1-propanol	14.55	500	1.93

[a]The K_m and V_{max} values for each substrate were determined by measuring the initial reaction rates at various nonsaturating concentrations of the substrate in the presence of a fixed volume of enzyme.

Effects of pH and Temperature on Activity and Stability of Enzyme. The enzyme activity toward *n*-hexanol was relatively stable at pH 3.0−4.0 and temperatures lower than 40 °C, while it decreased greatly beyond these ranges (Figure 5). The optimum conditions for the enzyme were obtained at pH 4.0 and 30 °C with a maximum specific activity around 150 μM min^{-1} (mg protein)$^{-1}$.

Effect of Metal Ions and Inhibitors on Enzyme Activity. Table 3 shows the results of metal ion effect and the inhibitory study, which was carried out by using different ions and inhibitors in the reaction mixture with *n*-hexanol as a

substrate. The enzyme activity was increased by Fe^{2+} but decreased by Mg^{2+} and Ga^{2+} at all tested concentrations (0.05−0.5 mM). Overall, the addition of K$^+$ and Zn$^+$ slightly decreased the enzyme activity, although a slight increase was found when these ions were added at a low concentration (0.05 mM) and a high concentration (0.5 mM), respectively. EDTA showed a significant decrease of enzyme activity at a higher concentration. When 1.0 mM EDTA was used, only 6.07% of enzyme activity was remained. Therefore, the enzyme might be metal ion-dependent in activity toward *n*-hexanol.

The addition of SDS, SAS, and DTT decreased the enzyme activity greatly, especially at a high concentration. GSH increased the enzyme activity at lower concentrations (lower than 0.5 mM) but caused a decrease at a high concentration (5.0 mM). The enzyme activity was increased in the presence of L-cysteine at high concentrations (0.5−5.0 mM). The inhibition effects of surfactants (SDS and SAS) and denaturants (DTT) on the enzyme activity were caused by the destruction of the enzyme.

Cofactor Effect. The cofactor experiments showed that the enzyme activity toward *n*-hexanol was increased in the presence of NADH but decreased in the presence of NAD$^+$, NADP$^+$, and NADPH (Table 4). According to the GC/MS analysis, *n*-hexane and *n*-hexanal were produced after the enzyme reaction on *n*-hexanol. Further study showed that the enzyme also had activities toward *n*-hexane and *n*-hexanal. The addition of NADH could also increase the enzyme activity toward *n*-hexanal. However, the enzyme activity toward *n*-hexane was inhibited by both NADH and NAD, and that toward *n*-hexanal was also inhibited by NAD (Table 5). Therefore, the enzyme activity toward *n*-hexanol was in two directions toward *n*-hexane and *n*-hexanal.

DISCUSSION

Novel Characteristics Distinguished the Enzyme from Reported Enzymes. The enzyme was compared with the reported alcohol dehydrogenase (ADH, EC1.1.1.1) since it showed activity toward alcohols. However, the characteristics of no activity toward ethanol and methanol, low optimum pH (pH 4.0), and NAD inhibition made the enzyme significantly different from most reported ADHs. ADH is widely distributed in mammals,[22,23] plants,[24,25] and microorganisms.[26,27] Apple ADH was also widely mentioned due to the fact that it plays an important role in the aroma volatile formation.[28] Almost all of the reported ADHs had activity toward ethanol, although some of them also had activities toward 1-butanol, 1-pentanol, and *n*-hexanol.[29,30] Most ADHs showed an optimum pH of 6.0−10.2 and NAD or NADP dependent.[31−39] The enzyme is also different from alcohol oxidases, since no reports revealed the effects of NAD and NADH on the activity of alcohol oxidases toward *n*-hexanol. Therefore, more research is needed to ascertain the class of the enzyme.

It is important to mention that the low optimum pH would indicate a great potential application of the enzyme in food processing. Paillard et al. found that the smashed apple tissue had capability on production of hexanal and 2-hexenal.[40]
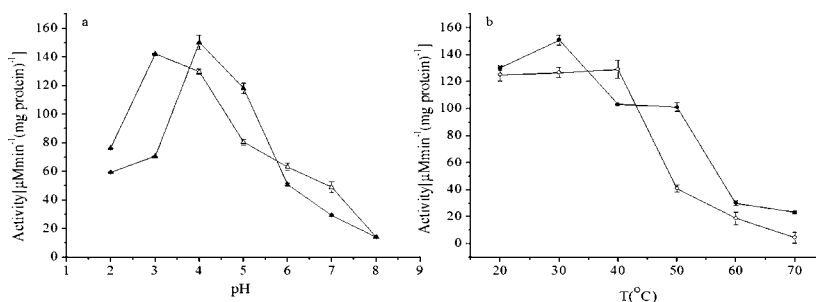
Figure 5. Effect of pH (a) and temperature (b) on the enzyme activity (closed triangle and closed circle) and stability (open triangle and open circle) toward n-hexanol. In the stability tests, the residual enzyme activity was measured at 30 °C and pH 4.0 after different treatments. In the activity tests, the enzyme activity was measured at 30 °C and different pH values in the pH treatments and at pH 4.0 and different temperatures in the temperature treatments. The bars in the curves show the standard deviations of triplicates.

Table 3. Effect of Different Metal Ions and Inhibitors on the Enzyme Activity toward n-Hexanol[a]

compd	concn (mM)	relative activity (%)	activity [μM min^{-1} (mg protein)$^{-1}$]
control	0	100	129.13 ± 0.53[b]
Ca^{2+}	0.05	17.77	22.95 ± 0.64
	0.1	80.54	104.00 ± 1.92
	0.5	40.83	52.72 ± 0.48
Fe^{2+}	0.05	129.23	166.88 ± 1.01
	0.1	147.38	190.31 ± 1.29
	0.5	120.28	155.32 ± 1.36
K^{+}	0.05	107.02	138.20 ± 1.88
	0.1	52.17	67.37 ± 0.89
	0.5	36.21	46.76 ± 0.31
Mg^{2+}	0.05	88.34	114.07 ± 1.20
	0.1	71.39	92.19 ± 0.59
	0.5	53.15	68.63 ± 0.93
Zn^{2+}	0.05	26.11	33.72 ± 0.15
	0.1	50.22	64.85 ± 0.82
	0.5	109.07	140.84 ± 0.95
EDTA	0.4	169.21	218.50 ± 0.14
	0.6	67.4	87.03 ± 0.44
	0.8	26.38	34.06 ± 0.16
	1.0	6.07	7.84 ± 0.02
L-cysteine	0.05	94	121.38 ± 0.41
	0.5	101.73	131.37 ± 0.69
	5	118.55	153.08 ± 0.31
SDS	0.05	66.14	85.41 ± 0.40
	0.5	59.82	77.25 ± 0.17
	5	57.43	74.16 ± 0.08
SAS	0.05	127.88	165.13 ± 0.64
	0.5	56.56	73.04 ± 0.05
	5	51.22	66.14 ± 0.86
DTT	0.05	144.9	187.11 ± 0.25
	0.5	58.75	75.86 ± 0.13
	5	26.21	33.85 ± 0.35
GSH	0.05	159.19	205.56 ± 2.77
	0.5	111.57	144.07 ± 1.05
	5	97.62	126.06 ± 0.54

[a]The enzyme activity was determined by incubating the enzyme in the presence of various compounds for 30 min at 30 °C and pH 4.0 with 9.8 mM n-hexanol as a substrate. [b]Standard deviation of triplicates.

Therefore, the crude enzyme might be directly used in food processing. Also, the enzyme could have great potential in the production of wine and alcoholic beverages (pH 2.7−3.8) since it had a high activity and stability at pH 2.0−4.0.

Table 4. Effect of Cofactors on the Enzyme Activity toward n-Hexanol[a]

cofactor	relative activity (%)	activity [μM min^{-1} (mg protein)$^{-1}$]
control[b]	100	110.89 ± 0.76[c]
NAD^{+}	36.59	40.58 ± 0.45
NADH	156.16	173.17 ± 1.32
NADP^{+}	89.52	99.27 ± 0.57
NADPH	56.47	62.62 ± 0.76

[a]The enzyme activity was determined by incubating the enzyme in the presence of different cofactors for 30 min at 30 °C and pH 4.0 with 9.8 mM n-hexanol as a substrate. [b]Without NAD^{+}, NADH, NADP^{+}, and NADPH. [c]Standard deviation of triplicates.

Table 5. Enzyme Activity toward Different Substances [μM min^{-1} (mg Protein)$^{-1}$]

cofactor	n-hexane	n-hexanol	n-hexanal
control[a]	39.20 ± 0.27[b]	110.89 ± 0.76	72.84 ± 0.94
NAD^{+}	0.62 ± 0.003	40.58 ± 0.45	67.08 ± 0.88
NADH	29.92 ± 0.16	173.17 ± 1.32	195.33 ± 1.19

[a]Without NAD^{+} and NADH. [b]The standard deviation of triplicates.

Enzyme Was Consistent with Alcohol Dehydrogenases in Metal Ion Effect and Inhibitors. The increase of enzyme activity by GSH (at low concentrations) and L-cysteine indicated that the sulfydryl group (−SH) might be essential for the enzyme activity, which was consistent with the reported findings.[41] The increase of the enzyme activity toward n-hexanol by the addition of Zn^{2+} was consistent with the reports that most of reported alcohol dehydrogenases contained zinc in subunits, including apple alcohol dehydrogenase.[42,43] This Zn ion plays a structural role and is crucial for protein stability. We also found that the enzyme activity toward n-hexanol could be reduced almost completely through inhibition by EDTA at a high concentration (1.0 mM). This is consistent with the ion dependence of alcohol dehydrogenase. The enzyme isolated from apple is sensitive to chelating agents, which might be due to the presence of zinc atoms at the active sites. However, more research is still needed to determine the structure of the enzyme.

It was also reported that a large group of metal ions deactivated ADHs, mostly by reacting with −SH residues of the enzymatic amino acid structure.[44] These ions may also interact with free carboxyl groups of the enzyme, thus altering the enzyme's conformation, which will also lead to partial deactivation of the enzyme. Another important category affecting the enzyme activities is related to chelates (e.g., EDTA), which influence the

activation of the enzyme by blocking a metal (usually zinc) and possessing a central place into the enzyme's active site.

We also found that Ca^{2+} could inhibit the enzyme activity at all concentrations (0.05−0.5 mM), while Fe^{2+}, Mg^{2+}, K^+, and Zn^{2+} increased the enzyme activity at different optimum concentrations. This is consistent with the effects of Fe^{2+} and Mg^{2+} on alcohol dehydrogenase from *Candida* sp.,[35] and the effect K^+ on the enzyme from *Thermus thermophilus*,[36] as well as that from most organisms, including *Desulfovibrio gigas*, *Saccharomyces cerevisiae*, and *Sulfolobus solfataricus*.[37,38,43]

In conclusion, a novel enzyme was extracted, purified, and characterized in this reported research. The enzyme showed high activity at pH 4.0 toward higher alcohols, but not ethanol and methanol, and thus indicated a potential in application for selectively decreasing the higher alcohol contents in foods. The enzyme was consistent with alcohol dehydrogenase in effect of metal ion and inhibitors but different in optimum pH, substrate specificity, and NAD(H) influence.

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Notes

The authors declare no competing financial interest.
Safety: No special safety problem needs to be emphasized.

■ ABBREVIATIONS USED

EDTA, ethylenediaminetetraacetic acid; NAD^+, nicotinamide adenine dinucleotide; $NADP^+$, nicotinamide adenine dinucleotide phosphate; GSH, glutathione; DTT, dithiothreitol; PEG, polyethylene glycol; SDS, sodium dodecyl sulfate; SAS, sodium aluminum sulfate

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