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Full Length Research Paper

Kinetic investigation on enantioselective hydrolytic resolution of epichlorohydrin by crude epoxide hydrolase from domestic duck liver

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Enantiopure epichlorohydrin is a valuable epoxide intermediate for preparing optically active pharmaceuticals. In this study, a novel epoxide hydrolase prepared from domestic duck liver was used as biocatalyst for producing (*S*) - epichlorohydrin from its racemates. To characterize the biocatalytic profiles of crude epoxide hydrolase, some effect factors were investigated. The crude epoxide hydrolase has an apparent optimal pH of 8.0 and an optimal temperature of 35°C. Fe^{2+} and Mn^{2+} both enhanced the activity of epoxide hydrolase to different degrees. The results of kinetic study revealed that a significant inhibition phenomenon is observed in this reaction process. The apparent kinetic parameters (*viz.*, V_{max} and K_m) and apparent substrate inhibition parameter K_{is} were calculated with linear fitting. The developed production process indicates that the novel epoxide hydrolase from domestic duck liver is a highly efficient biocatalyst for preparing enantiopure epichlorohydrin.

Key words: Epoxide hydrolase, enantiopure epichlorohydrin, enantioselective hydrolysis, kinetic resolution.

INTRODUCTION

Optically pure bioactive compounds are preferred over racemic mixtures because they are more targets specific and they show few, if any, undesirable side effects (Hwang et al., 2008; Liang et al., 2009). As a result, demand for these optically pure compounds is expected to extend in the future (Weijers and De Bont, 1999; Kasai and Suzuki, 2003). Enantiomerically pure epoxides such as epichlorohydrin (ECH) can serve as synthons in the preparation of *β*-blockers (Narina and Sudalai, 2007), *L*-carnitine (Kabat et al., 1997), and radiosensitizer (Hori et al., 1997). Therefore, it has attracted much attention in the development of methods for the synthesis of enantiopure epoxides. Various chemocatalytic and biocatalytic methods have been developed for preparing chiral epoxides (Tokunaga et al., 1997, Swaving and De Bont, 1998, Spelberg et al., 2004, and Li et al., 2006). Among the biocatalytic routes, there are several main methods to produce the enantiopure ECH. One method is chiral 2,3-dichloropropan-1-ol (2,3-DCP) cyclization reaction with base (NaOH), in which the chiral 2,3-DCP was obtained from enantioselective degradation of its racemates by *Alcaligenes* sp. and *Pseudomonas* sp. (Kasai et al., 1992a,b). However, racemic 2,3-DCP was an unavailable material and there is racemation phenomenon during the cyclization reaction. The other method which is kinetic resolution of racemic epoxides using an enantioselective hydrolysis reaction by an epoxide hydrolase (EH) (EC 3.3.2.3) might be comercially useful because obtaining chiral epoxides with high optical purities from relatively cheap and readily available racemates is possible (Santaniello et al., 1992, Shimizu

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Abbreviations: ECH, Epichlorohydrin; **2,3-DCP,** 2,3-dichloropropan-1-ol; **EH,** epoxide hydrolase; **DMSO,** dimethyl sulfoxide; **GC,** gas chromatography; **EDTA,** ethylene diamine tetra acetic acid.

and Kataoka, 1999; De Vries and Janssen, 2003). Several attempts for the production of chiral ECH from its racemates by microbial kinetic resolution have been reported. An enantio-selective EH from an actinomycetes source has been observed in a *Nocardia* H8, showing enantioselectivity in the hydrolysis of racemic ECH (Weijers and De Bont, 1991). Fungal epoxide hydrolysis has been described for *Aspergillus niger* (Choi et al., 1999) and *Rhodotorula glutinis* (Weijers, 1997; Kim et al., 2004; Lee, 2007). However, microbial cells include only a small amount of EH and often need to be induced to express this enzyme. We have previously described a novel crude EH prepared from domestic duck liver that has enantioselective resolution racemic ECH to produce (*S*)-enantiomer (Ling et al., 2009), an easier and more economical source of EH.

To date, several studies on the biocatalytic properties of EH from various microbes exist (Genzel et al., 2001; Doumèche et al., 2006). Unfortunately, less attention has been paid to studying the dynamic process of the EH catalyzing enantiopure ECH production. This process urgently requires further study. In this study, the research on the dynamic process of enantioselective hydrolyzing of racemic ECH catalyzed by the novel EH is reported.

MATERIALS AND METHODS

Reagents

Racemic ECH was purchased from Shenbo Chemical, Co., Ltd., (Shanghai, China). All other reagents used in the present work were purchased from Sinopharm Chemical Reagent Co. Ltd., (China), and were of analytical reagent (A. R.) grade.

Preparation of crude EH

Fifty grams of fresh liver pre-frozen at 0 – 4℃ was homogenized 4 times in a Waring blender for 30 s each time. The homogenate was poured into 200 ml acetone at -20℃ with stirring. When the suspension had settled, the precipitate was collected by filtration using a Büchner funnel under vacuum, washed with 100 ml cold acetone followed by 50 ml cold ether, and then dried in a refrigerator at 0 – 4℃ (Lu et al., 2006).

EH assay

The dry pellets of crude EH (0.6 g) were suspended in 20 ml 200 mmol/l phosphate buffer (Na_2HPO_4 – NaH_2PO_4) in 50 ml screw-cap bottles sealed with a rubber septum and heated in a temperature controlled heating water bath (Taicang Experimental Instrumental Co., Ltd., Jiangsu, China). The kinetic resolution was initiated by adding ECH as substrate. At selected time intervals, 0.1 ml aliquots were periodically withdrawn from the reaction mixture and diluted with an equal volume of methanol containing dimethyl sulfoxide (DMSO) as an internal standard, and analyzed by gas chromategraphy (GC) KR-9 capillary column to determine the conversion of ECH. In addition, 3 ml reaction samples were withdrawn and extracted with 2 ml diethyl ether. Then the diethyl ether fraction was mixed with 8 ml dichlormethane containing isopropyl ether as an internal standard, and analyzed by GC ZKAT-Chiral B column immediately to quantify the remaining ECH enantiomers and their enantiomeric excess (*e.e.*%). The *e.e.*% of (*S*)-ECH was calculated as expressed in Equation (1). In Equation (1), *S* and *R* represented mole of (*S*)-ECH and (*R*)-ECH, respectively. One EH activity unit (U) is defined as the biocatalyst activity required for the hydrolysis of 1 μmol of (*R*)-ECH per minute. All the enzymatic reactions were run in triplicate.

$$\textit{Enantioselective excess of (S)} - \textit{ECH} = \frac{S - R}{S + R} \times 100\% \quad (1)$$

Effect of pH and temperature

The effects of pH on the enzyme activity and *e.e.*% of (*S*)-ECH were investigated by varying the pH of the aqueous solution, in the range of 3.0 to 9.0 using 200 mmol/l phosphate buffer (pH 5.0 to 7.0), Tris-HCl (pH 8.0), and Gly-NaOH (pH 9.0). The reactions were carried out at 120 rpm in a temperature controlled heating water bath. The *e.e.*% of ECH was measured at five different reaction temperatures ranging from 20 to 65℃ for various time intervals. Samples were withdrawn from the reaction mixtures at selected time intervals and immediately analyzed by GC.

Effect of various metal ions and chelating agent

The effect of different metal ions on the crude EH activity was determined by the addition of the corresponding ion at a final concentration of 1.0 mmol/l to the reaction mixture, and assayed under the above mentioned conditions. The enzyme assay was carried out in the presence of $CaCl_2$, $CuSO_4$, $FeSO_4$, $MnSO_4$, $ZnCl_2$ and $NiCl_2$. Additionally, the effect of a chelator of divalent cations (ethylene diamine tetra acetic acid, EDTA) was also determined by preincubation with the enzyme solution for 30 min at 30℃ before the addition of substrate.

Determination of kinetic parameters

EH activity was determined at crude enzyme addition amount ranging from 0.02 to 0.16 g/ml and substrate concentrations from 12.76 to 255.20 mmol/l. The data was fitted with Michaelis-Menten equation, and apparent kinetic parameters V_{max} and K_m at various temperatures (10 to 40℃) were estimated by linear regression using Hanes-Woolf plot.

Conversion determination with gas chromatography

The conversion analysis was performed using a SP-6890 gas chromatography (Lunan Ruihong Ltd, Shandong, China) equipped with KR-9 capillary column (30 m × 0.32 mm × 1.0 μm). The injector and flame ionization detector temperature were 200 and 280℃, respectively. The oven temperature was held at 170℃, N_2 being the carrier gas (1.5 ml/min). The injected volume was 0.8 μl with a split ratio set at 80:1.

Enantioselectivity determination with gas chromatography chiral stationary phase

The enantioselectivity analysis was performed with ZKAT-Chiral B capillary column (20 m × 0.25 mm × 0.5 μm). The injector and flame ionization detector temperature were 150 and 250℃, respectively. The oven temperature was held at 100℃, N_2 being the carrier gas(1.1 ml/min). The injected volume was 0.4 μl with a split ratio set at 60:1.

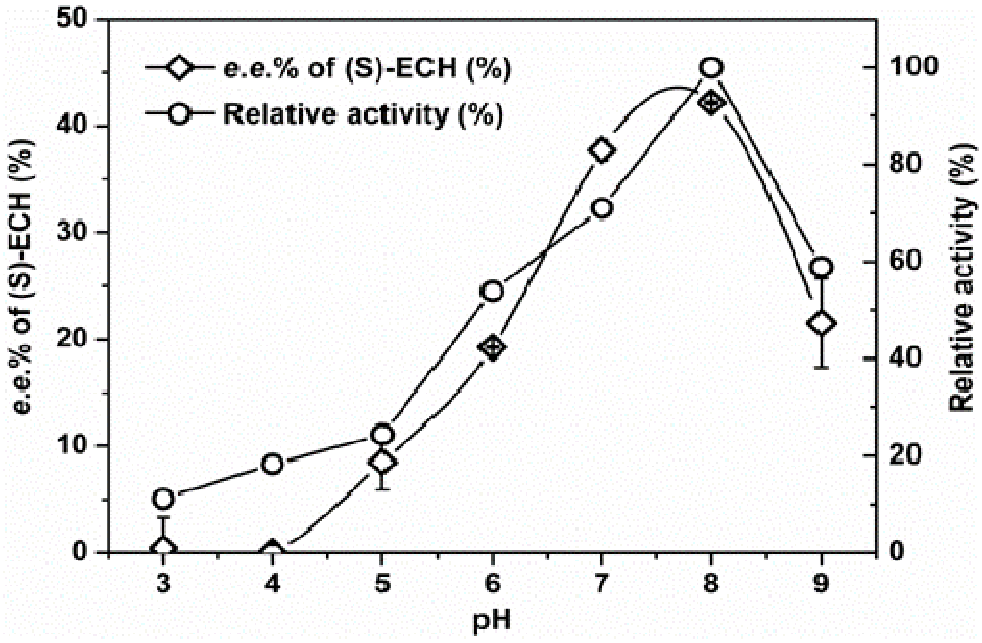

Figure 1. The influence of different pH on *e.e.*% of (*S*) -ECH and relative activity of EH.

RESULTS AND DISCUSSION

Effects of pH on relative activity of EH

The protein ionization state is an important determinant for enzyme catalysis. The effects of pH on EH activity and enantioselectivity were researched by changing the pH of the buffers mentioned earlier. The highest EH activity is found to be at pH 8.0 (Figure 1), with more than 58% of this activity in the pH range of 7.0 to 9.0. The *e.e.*% of (*S*)-ECH increases with pH from 3 to 8, and thereafter decreases from pH 8 to 9. These findings are in accordance with several earlier reports showing pH optima of 7.0 to 8.0 for partial purified EH from *A. niger* (Choi et al., 1999) and *R. glutinis* (Kim et al., 2004; Lee 2007), which also indicate that the EH from domestic duck liver is very sensitive to pH and lost a large degree of its activity when pH is lower than 5.0. EH has an optimum pH of about 8.0 at which their activity is maximal.

Effects of temperature on relative activity of EH

The effects of concentration of resolution reaction temperature and time influencing the *e.e.*% of (*S*)-ECH catalyzed by duck liver EH are shown in Figure 2. The highest *e.e.*% of (*S*)-ECH is at the resolution reaction temperature of 35℃ and time of 12 h. The curves of *e.e.*% of (*S*)-ECH between times at various temperatures indicate that reaction temperature and time are two significant parameters in the process of producing (*S*)-ECH. For the different reaction temperatures (20, 35, 45, 55 and 65℃), the *e.e.*% of (*S*)-ECH increases with reaction time, and reaches the maximal value when the reaction times are 12, 12, 10, 3 and 12 h, respectively. Then, the *e.e.*% of (*S*)-ECH decreases with the increase in reaction time. Within the temperatures investigated, at the same reaction time, such as 6 h, the *e.e.*% of (*S*)-ECH improves with rising temperature between 20 and 45℃, and declines with reducing temperature between 45 and 65℃ (Figure 2). However, with the increase of reaction time, the spontaneous hydrolysis speed of ECH without enantioselectivity rises. Therefore, selecting optimum hydrolysis temperature and time is essential to obtain maximum *e.e.*% of (*S*)-ECH. In the comprehensive consideration of the hydrolysis temperature and time, 20 to 45℃ and 8 to 12 h are the most suitable conditions for the production of enantiopure ECH from its racemates.

Effects of various compounds on enzyme activity

Enzyme activity was measured after incubation of the enzyme at 30℃ for 30 min with 1 mmol/l of various compounds (Figure 3). Some kinds of metal salts such as $CaCl_2$, $CuSO_4$, $ZnCl_2$ and $NiCl_2$, inhibit the enzyme activity. $CaCl_2$ decreases the enzyme activity to 95.9%, while $CuSO_4$, $ZnCl_2$ and $NiCl_2$ decrease it to 83.4, 93.9 and 88.0%, respectively. Conversely, $FeSO_4$ and $MnSO_4$ enhance the activity to 123.5 and 108.8%, respectively; this is possible because of the activation by the metal ions. EDTA also shows a negligible effect on enzyme activity with 97.6%. This result may be due to the EDTA bound to some inhibitory metal ions in the crude enzyme preparation.

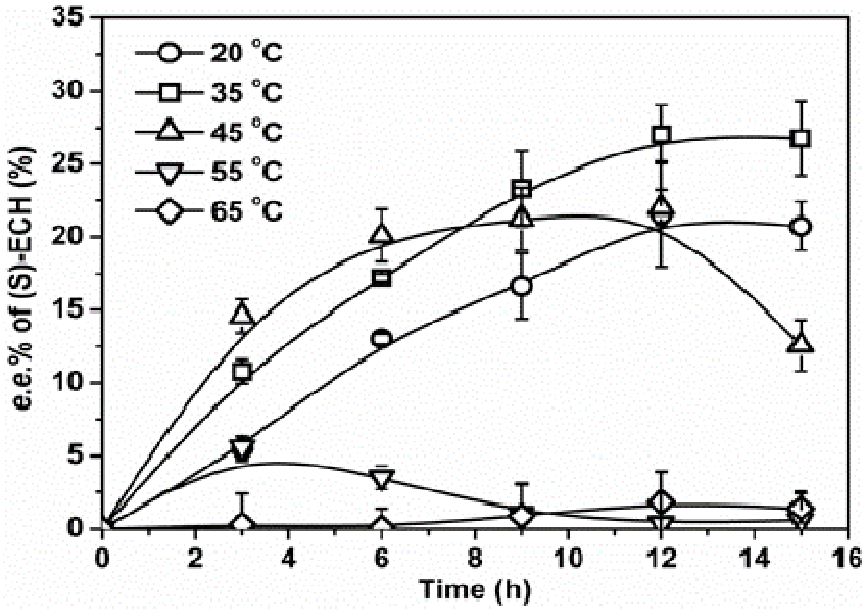

Figure 2. The influence of different reaction temperature and time on *e.e.*% of (*S*)-ECH.

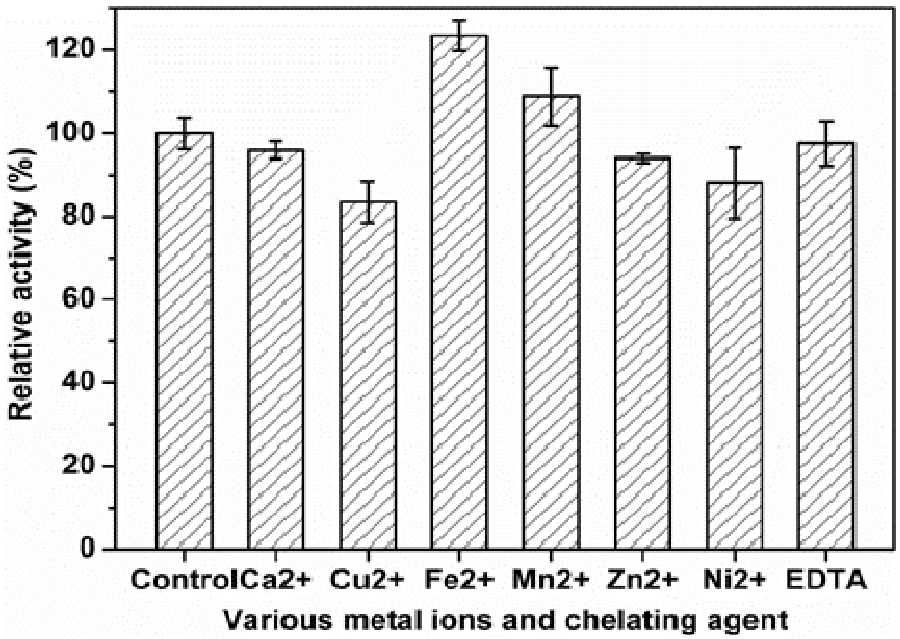

Figure 3. The influence of various metal ions and chelating agent on relative activity of EH.

Effects of addition amount of crude EH on initial rate

The crude EH pellets were incubated in the phosphate buffer (0.2 mol/l, pH = 7) with concentrations ($[E_0]$) from 0.02 to 0.16 g/ml at 30°C for 30 min. Then, racemic ECH was added into the reaction mixture with a concentration of 25.92 mmol/l equivalently. The effect of concentration of crude EH on the initial rate (v_0) is represented in Figure 4. The initial rate increases steeply with EH concentration when enzyme concentration is lower than 0.12 g/ml. On the other hand, the initial rate increases slightly when enzyme concentration is higher than 0.12 g/ml. At this time, the ratio of enzyme activity to substrate amount is 457:1 U/mol. As mentioned earlier, if the reaction rate is in accordance with Michaelis-Menten equation, equation $v_0 = k_2, [E_0] = V_m$ will hold under the condition of initial

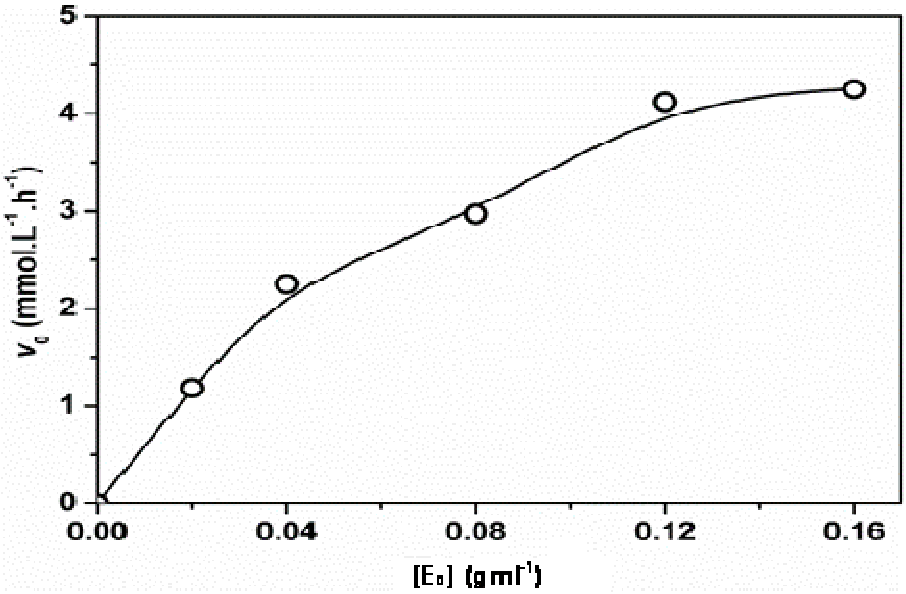

Figure 4. The influence of concentration of EH on the initial rate.

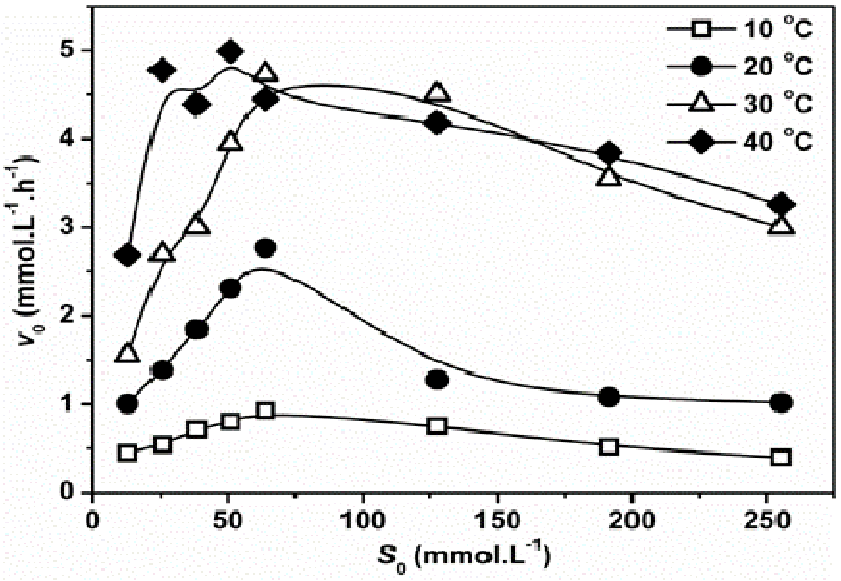

Figure 5. Effect of substrate concentration on the initial rate.

substrate concentration [S_0] >> K_m, that is, v_0 is directly proportional to [E_0]. However, v_0 shows nonlinear relationship with [E_0] as a fact in this reaction system, indicating that there is a significant inhibition phenomenon in this reaction process.

Effects of substrate concentration on initial rate and determination of kinetic parameters

Figure 4 also demonstrates that, the concentration of EH influences the reaction initial rate significantly. Therefore, the concentration of EH was kept at 0.06 g/ml constantly in the following experiments. Racemic ECH was added into the reaction mixture with concentrations ([S_0]) from 12.76 to 255.20 mmol/l. The kinetic resolution reactions were carried out at different temperatures for 2 h. Figure 5 depicts the effect of substrate concentration on initial rate at various temperatures. The effect shows that, for all four temperature levels (*viz*., 10, 20, 30 and 40°C), initial reaction rate increases with substrate concentration from 12.76 to 63.80 mmol/l. The rate then declines with the

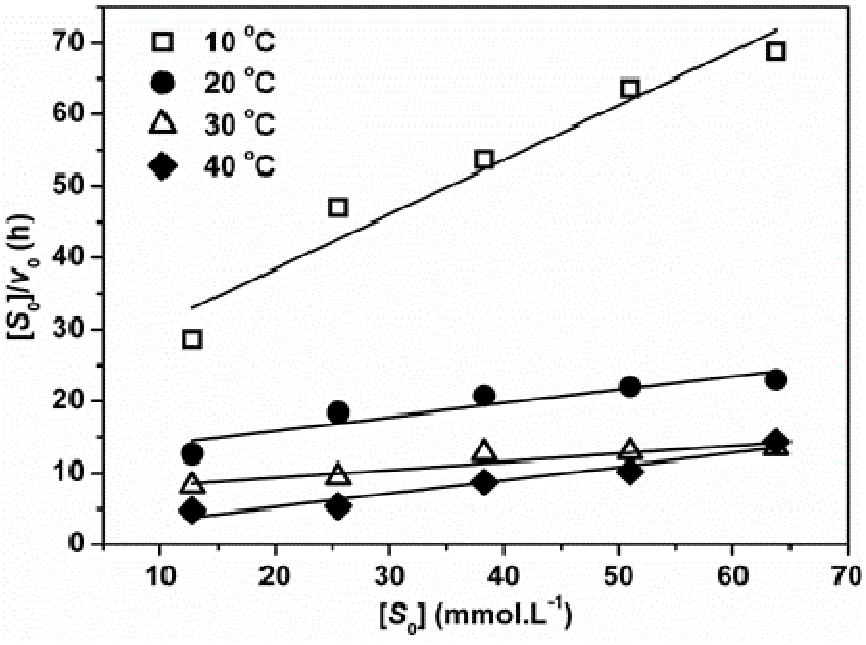

Figure 6. The relationship between $[S_0]$ and $[S_0]/v_0$ at low substrate concentration.

Table 1. Apparent kinetics parameters at different temperatures.

T (℃)	K_m (mmol/l)	V_m (mmol/(L·h))
10	30.550	1.315
20	63.194	5.236
30	65.415	9.124
40	7.740	5.308

enhancement of the substrate concentration at 63.80 to 255.20 mmol/l, which also confirms that a notable substrate inhibition indeed exists in this enzyme-catalyzed reaction.

When substrate concentration is at a low level ($[S_0]$ < 63.80 mol/l), the enzyme-catalyzed process is not inhibited. Consequently, the kinetic parameters of this process can be calculated with Michaelis-Menten equation shown in Equations (2) and (3) as follows:

$$v_0 = V_{max}[S_0]/(K_m + [S_0]) \quad (2)$$

$$[S_0]/v_0 = [S_0]/V_{max} + K_m/V_{max} \quad (3)$$

In Equation (3), the slope and intercept, namely, $1/V_{max}$ and K_m/V_{max} at each temperature, were fit-plotted with $[S_0]/v_0$ as ordinate against $[S_0]$ as abscissa (Figure 6). The fit-plotting reveals that the linear fitting agrees well with the experimental data, demonstrating that the reaction process accords with the Michaelis-Menten equation at the low substrate concentration. Table 1 depicts the apparent kinetic parameters V_{max} and K_m calculated with linear fitting. We can summarize from Figure 6 that V_{max} and K_m increases with temperature enhancement from 10 to 30℃, indicating that the dissociation rate of enzyme substrate complex ES is higher than its formation rate with temperature increase. On the other hand, V_{max} and K_m decline when temperature is below 30℃, which may be due to deactivation of the enzyme at this higher temperature.

As mentioned earlier, a significant substrate inhibition during the enzyme-catalyzed process at high substrate concentration ($[S_0]$ > 63.80 mol/l) is observed. Therefore, the rate of reaction can be described in Equations (4) and (5) as follows:

$$v_0 = V_{max}[S_0]/(K_m + [S_0] + [S_0]^2/K_{is}) \quad (4)$$

$$1/v_0 = 1/V_{max} + K_m/(V_{max}[S_0]) + [S_0]/(V_{max}K_{is}) \quad (5)$$

Furthermore, if substrate concentration $[S_0]$ is 10-folds higher than K_m, (that is, $[S_0]/K_m$ > 10), no products are added into the reaction system. Equations (4) and (5) can be simplified to Equation (6) as follows:

$$1/v_0 = [S_0]/(V_{max}K_{is}) + 1/V_{max} \quad (6)$$

As seen in Equation (6), the slope and intercept, namely $1/(V_{max}K_{is})$ and $1/V_{max}$ at 20 and 30℃, were fit-plotted with $1/v_0$ as ordinate against $[S_0]$ as abscissa (Figure 7). The linear fitting agrees well with the experimental data. The apparent kinetic parameter V_{max} at 20 and 30℃ was

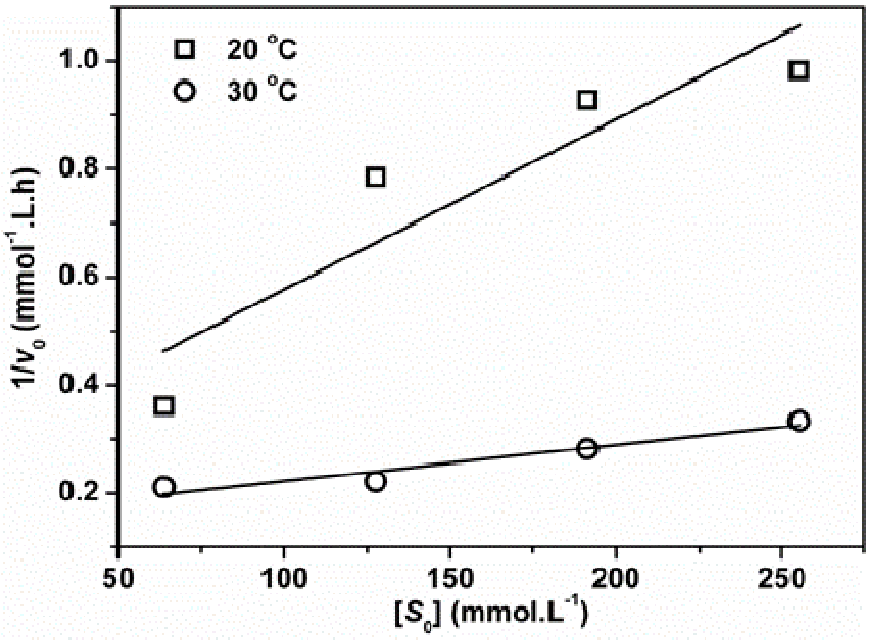

Figure 7. The relationship between initial rate and substrate at high concentration.

calculated as 3.81 and 6.44 mmol/(L·h), and apparent substrate inhibition parameter K_{is} at 20 and 30°C as 84.58 and 222.07 mmol/l, respectively.

Conclusion

A novel EH from domestic duck liver was utilized to catalyze enantioselective hydrolysis kinetic reaction of racemic ECH to produce its (*S*)-enantiomer. The enzymatic reaction conditions were optimized. The highest EH activity is found to be at pH 8.0. The highest *e.e.*% of (*S*)-ECH is at 35°C and 12 h. In addition, $FeSO_4$ and $MnSO_4$ enhanced the activity of EH to 123.5 and 108.8%, respectively. In the kinetic studies, the initial rate increases steeply with EH concentration when enzyme concentration is lower than 0.12 g/ml, but increases slightly during further enhancement of enzyme concentration. This nonlinear relationship between v_0 and $[E_0]$ indicates that a significant inhibition phenomenon exists in this reaction process. The apparent kinetic parameters (*viz.*, V_{max} and K_m) and apparent substrate inhibition parameter K_{is} were determined with linear fitting. The developed production process indicates that the novel EH from domestic duck liver is a preferable biocatalyst that can be used for enantioselective resolution of racemic ECH.

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