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Facile synthesis of chiral 2-hydroxy acids catalyzed by a stable duck ε-crystallin with endogenous L-lactate dehydrogenase activity

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Duck ε-crystallin, an abundant structural protein in lenses of some avian species, was shown to possess a genuine and stable L-lactate dehydrogenase (L-LDH; EC 1.1.1.27) activity suitable for the application to enzyme technology as a catalyst for the synthesis of chiral α-hydroxy acids. Two pharmaceutically important intermediates, 2-hydroxy acids (*S*)-2-hydroxybutanoic acid (*S*)-2-hydroxypentanoic acid) have been synthesized in high yields and optical purity utilizing an in situ NADH regeneration system of duck ε-crystallin coupled with formate/formate dehydrogenase. This enzyme system is also shown to offer some advantages over the conventional L-LDH sources from several mammalian species.

Lens crystallin; Lactate dehydrogenase; Chiral synthesis; 2-Hydroxybutanoic acid; 2-Hydroxypentanoic acid; Duck

1. INTRODUCTION

Bioorganic synthesis based on enzymatic catalysis is providing an increasingly important tool in the methodology of enantio-selective synthesis of valuable organic intermediates (i.e. chiral compounds) [1,2]. Despite many practical applications of enzymes for the generation of chiral compounds, not many studied enzymes simultaneously possess broad substrate specificity and high enantio-selectivity. However, L-LDH was previously shown to be one of the efficient catalysts that should be useful in the enantio-selective reduction of unnatural-2- oxo acids [3].

Recent crystallin studies have revealed that one of the structural lens proteins, i.e. ε-crystallin isolated from the avian classes, appeared to be structurally and functionally similar to heart-type lactate dehydrogenase, based on sequence comparison [4] and kinetic analysis [5,6]. This crystallin is especially rich in the lenses of birds belonging to the family, Anatidae, which includes ducks, swans and geese [7]. It comprised about 10–20% of total protein in the duck lens and could be isolated easily by single-step purification [8]. Here we report the application of duck ε-crystallin, an abundant natural mutant of LDH with high stability, for the synthesis of two chiral 2-hydroxy acids, (*S*)-2-hydroxybutanoic acid and (*S*)-2-hydroxypentanoic acid [9]. By utilizing the inexpensive and stable ε-crystallin with endogenous LDH activity, coupled with an in situ NADH regeneration system of formate/formate dehydrogenase, these pharmaceutically useful chiral compounds have been synthesized with high yields and high optical purity, thus circumventing the use of relatively expensive conventional LDHs or other organic synthesis approaches. This should prove a great potential in the asymmetric synthesis of some other biosynthetic intermediates.

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2. MATERIALS AND METHODS

2.1. *Enzyme sources and reagents*

The duck (a cross-bred hybrid between *Cairina moschata* and *Anas platyrhynchos* var. *domestica*) lenses were obtained from the Taiwan Livestock Research Institute, Yinan, Taiwan. NADH, 2-oxobutanoic acid and 2-oxopentanoic acid were obtained from Sigma Chemical Company (St. Louis, MO, USA). Formate dehydrogenase (FDH; from yeast, EC 1.2.1.2.) was obtained in lyophilized form from Boehringer-Mannheim (Mannheim, Germany). Enzymes used for kinetic measurements were dissolved in 30 mM phosphate-buffered solution. Chemical shifts of ^{1}H NMR and ^{13}C NMR spectra were recorded with a Bruker AM-200 using solvent peaks as references. IR spectra were recorded from KBr thin films with a JASCO IR-700. UV absorbance was measured in a Hitachi U-2000 spectrophotometer. The optical rotation was measured using a Universal Polarimeter (Schmidt & Haensch, Berlin, Germany). The HPLC system was composed of two Waters Model 6000 pumps, a Waters MOdel 450 UV detector, and an M-660 solvent programmer. High purity solvents were obtained from ALPS Chemical Company, Taiwan.

2.2. *Enzyme preparation*

The batch preparations of crystallins were routinely carried out from the pooled lenses of about 10 duck eyeballs. They were freshly collected from the above Institute without freezing, decapsulated, and homogenized in 20–30 ml of 0.05 M ammonium bicarbonate buffer, pH 7.7, containing 5 mM EDTA. The clear solution of the crude homogenate from centrifugation was lyophilized to give 200 mg of the crude enzyme powder which was used directly without further purification. The purification of ε-crystallins from the crude extract was essentially according to the previous report on a TSK DEAE-650(M) anion-exchange column [5].

2.3. *Kinetics of ε-crystallin*

The kinetic parameters of ε-crystallin were measured according to the previous report [3]. The L-lactate dehydrogenase activities of ε-crystallin were studied in the direction of reduction in the presence of NADH in 30 mM phosphate buffer (pH 7.2) at 25°C by measuring the decrease in UV absorbance at 340 nm. The assay solution containing NADH (0.2 mM) and various concentrations of substrates plus various amounts of ε-crystallin was dissolved in 30 mM phosphate buffer (pH 7.2) before use. The concentrations of substrates were increased from 0.2 to 5 K_m, and the amount of ε-crystallin was increased by a factor of 100-fold. The kinetic parameters were obtained from Eadie–Hofstee plots.

2.4. *Stability of ε-crystallin*

The stabilities of ε-crystallin under synthetic conditions were measured spectrophotometrically by measuring the absorbance change at 340 nm due to NADH consumption in the LDH reaction. In a typical measurement, ε-crystallin (0.56 mg) in phosphate buffer (100 ml) was stirred at 35°C to maintain homogeneity. Periodically, 1.0 ml aliquots were taken and added to a cuvette containing NADH (200 μM), pyruvate (200 μM) in 0.2 M phosphate buffer. The initial reaction rates were determined from time-dependent plots of the decreasing absorbance at 340 nm.

2.5. *Synthesis of (S)-2-hydroxypentanoic acid and (S)-2-hydroxybutanoic acid*

To a solution of sodium formate (1.7 g, 25 mmol), sodium 2-oxopentanoate (2.76 g, 20 mmol) and β-mercaptoethanol (100 μl) dissolved in water (30 ml) was added NAD^+ (66.0 mg, 0.1 mmol), ε-crystallin (80 U) and formate dehydrogenase (FDH, 8 U). The resulting solution was flushed with nitrogen for 30 min. The pH of reaction solution was adjusted to 7.5 and kept constant between 7.4–7.6 throughout the synthesis with a pH-controller by adding 1.0 M HCl. After the reaction was complete (4.5 days), the solution was acidified to pH 2.0 with 6 M HCl and extracted with diethyl ether (3 × 100 ml). The combined ether solution was dried over anhydrous Na_2SO_4 and concentrated on a rotatory evaporator to give an oily residue which was solidified after further drying in vacuo. The yield was 2.19 g (18.4 mmol, 93%). Using the same procedure, from sodium 2-oxobutanoate (2.48 g, 20 mmol), β-mercaptoethanol (0.1 ml), NAD^+ (66 mg, 0.1 mmol), ε-crystallin (80 U), sodium formate (1.7 g, 25 mmol) and FDH (8 U), (*S*)-2-hydroxybutanoic acid (1.93 g, 18.4 mmol, 92%) was obtained. The physicochemical and spectroscopic data are shown as follows:

(S)-*2-hydroxybutanoic acid:* mp 49–50°C (Lit. mp 54.5–55.5°C in [3]); $[\alpha]_D^{25}$ = +6.4 (c 2.03%, $CHCl_3$) (Lit. $[\alpha]_D^{25}$ = +6.4 (c 2.63%, $CHCL_3$) in [10]); ^1H NMR ($CDCl_3$, 200 MHz), δ: 6.54 (br s, 2 OH), 4.24 (dd, J = 7 Hz, J' = 4 Hz, 1 H), 1.88 (m, 1 H), 1.76 (m, 1 H), 1.00 (t, J = 7 Hz, 3 H) ppm; ^{13}C NMR ($CDCl_3$), δ: 179.5, 71.2, 27.2, 8.9 ppm; IR(KBr), 1128, 1217, 1723, 2971, 3410, 2500–3700 cm^{-1}.

(S)-*2-hydroxypentanoic acid:* mp 53–55°C (Lit. mp 52–55°C in [10]); $[\alpha]^{25}$ = −2.69 (c 4.8%, H_2O) (Lit. $[\alpha]^{25}$ = −2.7 (c 1–5%, H_2O) in [11]); ^1H NMR ($CDCl_3$, 200 MHz), δ: 6.76 (br, 2 H), 4.26 (d,d, 1 H), 1.78 (m, 1 H), 1.67 (m, 1 H), 1.46 (m, 2 H) 0.93 (t, 3 H) ppm; ^{13}C NMR ($CDCl_3$) δ: 179.8, 70.1, 36.1, 18.0, 13.6 ppm; IR(KBr) 1132, 1222, 1723, 2960, 3410, 2400–3800 cm^{-1}.

The pharmaceutical use of these two chiral compounds as intermediates in the synthesis of various insect pheromones and fungicides has been mentioned ([3,9,10] and references cited therein).

3. RESULTS

ε-Crystallin, a relatively abundant lens protein present in some avian and reptilian species, was shown to possess an L-lactate dehydrogenase activity with high stability suitable for application to enzyme technology [5]. Lactate dehydrogenase is a 'house-keeping' nicotinamide-dependent enzyme of glycolysis, the major metabolic pathway that metabolizes glucose to lactate and generates the energy in the form of ATP in the absence of oxygen for most organisms [12]. The presence of the specific distribution of different LDH isozymes in various tissues does not seem to be a random occurrence but is of functional importance in tissue metabolism and has developed from natural selection [13]. Many methods for L-LDH immobilization have been documented to improve the stability of L-LDH for chiral synthetic purpose [14,15]. A convenient in situ NADH regeneration system for the LDH reduction reaction has also been developed to enhance the capability of LDHs for preparative scale synthesis [16]. We report here the first successful application duck ε-crystallin with L-LDH activity as a catalyst for chiral 2-hydroxy acid synthesis.

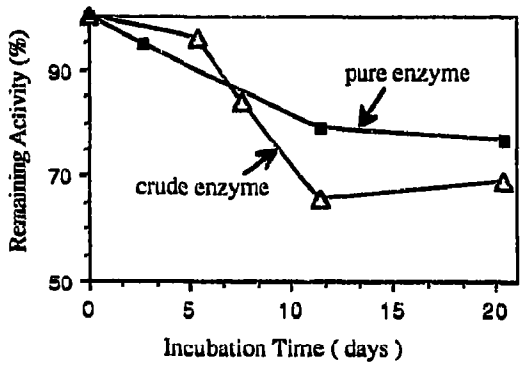


Fig. 1. Stability of duck crude extract and purified ε-crystallin in aqueous solution at pH 7.5 and 25°C. ε-Crystallin (0.56 mg) and crude homogenate (1.0 mg) each in phosphate buffer (100 ml) were stirred at 35°C to maintain homogeneity. Periodically, 1.0 ml aliquots were taken and added to a cuvette containing NADH (200 μM), pyruvate (200 μM) in 0.2 M phosphate buffer, pH 7.5. The stabilities were studied spectrophotometrically by measuring initial reaction rates as reflected in the absorbance change at 340 nm due to NADH consumption in the LDH reaction at 25°C.

ε-Crystallin was prepared from the pooled lenses of eyeballs (10 balls, 900 mg), homogenized and centrifuged to give a crude lyophilized enzyme preparation (200 mg) which has a specific activity of about 100–120 U for reduction of pyruvate. Further purification by a DEAE ion-exchange chromatography gave a specific activity of 610 U. Therefore isolation and purification of ε-crystallin was very easy as compared to the conventional purification of various LDHs from other tissues [17]. The structures of these products were confirmed by ^1H NMR, ^{13}C NMR, IR and optical rotation data. They were obtained in high yields (92 and 93%) and optical purity (>99% for both products). From an HPLC analysis of the reaction product in each time interval, more than 90% of the product was formed in the first half of the total reaction period. In this study the L-lactate dehydrogenase activity associated with the ε-crystallin of duck lens is found to be very stable ($t_{1/2}$ at pH 7.5 and

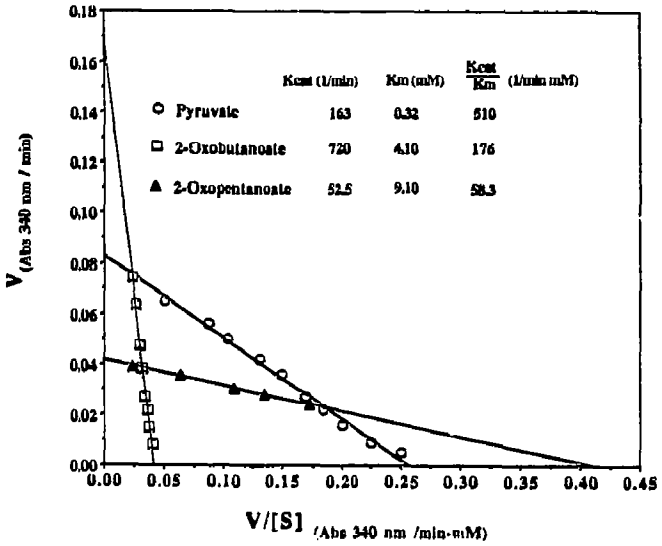


Fig. 2. Determination of kinetic parameters by Eadie–Hofstee plots for the reduction of three 2-oxo acids catalyzed by purified duck ε-crystallin. The L-lactate dehydrogenase activities of ε-crystallin were studied in the direction of reduction in the presence of NADH in 30 mM phosphate buffer (pH 7.2) at 25°C by measuring the decrease in UV absorbance at 340 nm. The assay solution containing NADH (0.2 mM) and various concentrations of substrates was dissolved in 30 mM phosphate buffer (pH 7.2) before use. The concentrations of substrates were increased from 0.2 to 5 K_m, and the amount of ε-crystallin was increased by a factor of 100-fold.

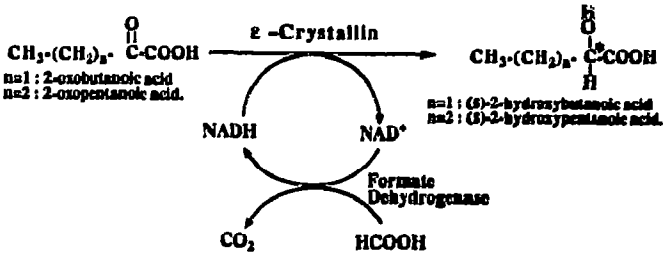
room temperature is more than twenty days and about 4 h at 62°C).

Fig. 1 shows the stability of crude and purified ε-crystallin in phosphate buffer (pH 7.5) at 25°C. The activity of an aliquot of the reaction mixture 30 s after addition of the ε-crystallin solution was taken as 100% activity. The rate of inactivation of the enzyme was measured on the basis of the remaining activity (using the activity of 30 s incubation of ε-crystallin as the reference activity of 100%). The enzyme can maintain its activity in both phosphate buffer and in pure water. The latter condition is more suitable for large-scale synthesis because the mixture of enzymes, coenzyme, and substrates in solution can serve as a buffer. As shown in Fig. 1, after a 21-day incubation, both crude and purified enzyme still possessed at least 65% of their original activity. Fig. 2 shows the kinetic parameters of ε-crystallin for the LDH-catalyzed reduction of pyruvate, 2-oxobutanoate, and 2-oxopentanoate. The enzyme has a specific activity of 610 U/mg and k_{cat} of 720 min^{-1} and 53.5 min^{-1} for (*S*)-2-butanoic acid and (*S*)-2-pentanoic acid, respectively. The k_{cat} value of 2- oxobutanoate is 14-fold higher than that of 2-oxopentanoate.

4. DISCUSSION

Most of L-LDHs used for synthetic application must possess a high specific activity and fair stability

Scheme 1



Scheme 1. ε-Crystallin-catalyzed synthesis of (*S*)-2-hydroxy acids by an in situ NADH regeneration system coupled with formate/formate dehydrogenase.

[3,10,18,19]. As shown in the previous report [5,6] regarding the stability and specific activity shown here ε-crystallin actually exhibited better stability and higher specific activity than the commercial LDHs isolated from different tissues of various animal species [3]. Comparison of kinetic data for ε-crystallin with those of other known LDHs used previously in asymmetric synthesis also reveals that the ε-crystallin has a higher k_{cat} than the catalytic constants of all the others [3,18].

Scheme 1 shows the overall reactions for the generation of 2-hydroxy acids utilizing the L-LDH catalyzed reactions in coupling with a formate/formate dehydrogenase system. The rate limitation is dependent on the activity of formate dehydrogenase, i.e. increasing the amount of formate dehydrogenase can shorten the time for reaction completion. Similar results are obtained when using the same units of purified ε-crystallin or crude crystallin extract as catalyst.

In summary, the procedure illustrated here describes the practical application of ε-crystallin in the preparative-scale enzymatic synthesis of optically pure 2-hydroxy acids. Since the ε-crystallin is a naturally abundant structural protein (10–20% of total lens protein) present in some avian lenses it could prove to be more economical than adopting conventional LDHs from heart or muscle tissues, which usually are present in much lower quantity. In addition the kinetic parameters shown for duck ε-crystallin are also superior to those of conventional LDHs regarding the crucial catalytic properties for biosynthetic purposes.

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