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NEW INHIBITORS FOR DICARBONYL/L-XYLULOSE REDUCTASE IN *CAENORHABDITIS ELEGANS*

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SUMMARY

Dicarbonyl/L-xylulose reductase (DCXR) is an enzyme reducing dicarbonyl (-CO-) groups in single sugar molecules and participates in the sugar metabolism of several sugar metabolism cycles in different living organisms (humans, other animals, fungi, and microorganisms). *Caenorhabditis elegans*, a model organism, has a unique DCXR (referred to as Ce DCXR) which plays a biochemical function similar to its homologs in the other organisms. However, the catalytic regulation of the enzyme has not been fully elucidated yet. Therefore, in this study, we investigated the chemicals which could inhibit the enzyme and found three inhibitor compounds including hexanoic acid, phosphoenolpyruvic acid, and DL-α-aminobutyrate hydrochloride for the enzyme. Because these inhibitors and their derivatives suppressed DCXRs in humans, mice, rabbits, and rats, they possibly inhibit the DCXR homologs at least in other animals. If the inhibitors and their derivatives are components in pharmaceutical products, foods, and drinks, they may come into cells, interact with DCXRs and inactivate them, causing toxicity for the host organisms.

Keywords: *Caenorhabditis elegans*, DCXR, *dhs-21* gene, hexanoic acid, inhibitor, methyl-DL-α-aminobutyrate hydrochloride, phosphoenolpyruvic acid, recombinant protein

INTRODUCTION

Dicarbonyl/L-xylulose reductase (DCXR; EC 1.1.1.10) catalyzes the reduction of a variety of simple sugar molecules and L-xylulose bearing two carbonyl groups (-CO-) to alcohol groups (-CHOH) in many different species (Nakagawa *et al.*, 2002; Richard *et al.*, 2002; Ishikura *et al.*, 2003; Matsunaga *et al.*, 2008). The structures of the DCXR homologs in different species have high similarity (Doten, Mortlock 1985; Mobus, Maser 1998; Richard *et al.*, 2002; Ishikura *et al.*, 2003; Zhao *et al.*, 2009; Son le *et al.*, 2011).

The carbonyl group is highly reactive in free sugar molecules, which can react with other molecules (amino acids of proteins, lipids, etc.) without the participation of a catalytic enzyme (called Maillard Reaction (MR)) to form advanced glycation end-products (AGEs) (Shen *et al.*, 2020). AGEs disrupt cellular metabolisms

through the malfunctions of macromolecules in the cells. DCXRs are active to carry out the catalysis of the dicarbonyl groups of sugar molecules, so the amount of cellular AGEs is not accelerated in the cells (Asami *et al.*, 2006; Odani *et al.*, 2008). In humans, DCXRs are found to have more functions. It catalyzes the reduction of reactive oxygen species (ROS) which trigger apoptosis (Matsunaga *et al.*, 2008; Singh *et al.*, 2009). The overexpression of DCXR in prostate cells is a positive indicator for the medical diagnosis of prostate cancers (Cho-Vega *et al.*, 2007).

In our previous research, Ce DCXR of the model nematode *C. elegans* was reported to be encoded by a single gene *dhs-21* and essential for normal longevity and reproduction. The obtained results suggested that Ce DCXR plays a critical role that could not be compensated by any other enzymes in the development of *C. elegans*. However, the functional regulation of Ce DCXR and its homologs remain to be determined. We wanted to investigate the chemicals which could inhibit the enzymatic function of DCXR. Therefore, in this research, we produced the recombinant Ce DCXR and used it to test for the possible inhibition of three natural compounds (hexanoic acid, phosphoenolpyruvic acid, and methyl-DL-α-aminobutyrate hydrochloride). We found that three compounds could in part inhibit Ce DCXR, indicating that they may inhibit DCXRs in other animals.

MATERIALS AND METHODS

Chemicals

Methyl-DL-α-aminobutyrate hydrochloride (CAS No.: 7682-18-0, Sigma-Aldrich), hexanoic acid (CAS No.: 142-62-1, Sigma-Aldrich), NADPH (CAS No.: 100929-71-3, Sigma-Aldrich), phosphoenolpyruvic acid (CAS No.: 10526-80-4, Alfa Aesar) and 2,3-heptanedione (CAS No.: 96-04-8, Alfa Aesar), erythrulose (56845-5mL, Sigma-Aldrich), 1,4-dibromo-2,3-butanedione (D39169-25G, Sigma-Aldrich)

Production of recombinant Ce DCXR

Preparation of RNA library from the worm lysate (Ly *et al.*, 2015)

Total RNA was isolated and purified from *C. elegans* N2 by the chloroform method as following: 1) Gravid adult worms cultured on 05 Petri plates (10 cm) were washed off the plates with M9 buffer and put in a 2-mL tube; 2) The worms were settled down by gravity and washed again with M9 buffer 5 times; 3) The worm settled down by centrifuging at 6000 rpm and M9 buffer was quickly aspirated off, and 1 mL of Trizol was added to the tube; 4) The worm tube was flash-frozen in liquid N2 and thawed at 37ºC, repeating 7 times until the worm culture became a worm supernatant; 5) 200 µL of chloroform was added in the mixture tube (total 1 mL), the tube was then inverted up and down for 10 seconds, incubated at room temperature (~25ºC) for 3 min, and centrifuged at 12,000 rpm at 4ºC for 15 min; 6) Approximately 500 µL of the upper aqueous phase was transferred to a new 2 mL tube, and then the tube was added 500 µL of cold isopropanol. The solutions were mixed thoroughly and incubated at room temperature for 10 min; 7) The tube was centrifuged at 12,000 rpm at 4ºC for 10 min, and the total RNA pellet appeared, as a little clear RNA precipitate; 8) The supernatant (almost isopropanol) was removed and the RNA pellet was washed with 1 mL of cold 75% ethanol 3 times; 9) The RNA pellet was quickly dried at 60ºC; 10) 100 µL of RNase-free water was added in the tube with the RNA pellet and the tube was incubated at 37ºC for 1 hour; 11) The purification of the RNA solution was qualified for $A_{260/280}$ of more than 1.8 in a UV spectrometer, and RNA concentration was estimated by A_{260} (A_{260} of 1.0 = 33 to 40 ng/mL). The total extracted RNA solution was stored at -86ºC for cloning within 3 days.

Generation of Ce DCXR cDNA (Shaham, 2006)

The total extracted RNA solution was used to synthesize total cDNAs. A mixture of 8 µL of 25 mM $MgCl_2$, 4 µL of 10X buffer, 4 µL of dNTP mixture, 2 µL of ribonuclease inhibitor, 4 µL of

reverse transcriptase, 8 µL of 50 ng/µL total extracted RNA, 4 µL of oligo dT_{15}, 6 µL of nuclease-free water in a 40 µL RT-PCR reaction was incubated for 30 min at 42°C and then 95°C for 5 min. Next, the cDNA of *Ce DCXR* was amplified by PCR using specific a primer pair (F: 5'-GGAATTCCATATGCCCGCCAATTACGA TTTC, and R: 5'-CGCTCGAGTTAGTTATTCG AAAATCCTCCATC-3'). The PCR reaction was conducted in a 50 µL mixture solution including 2 µL of dNTP mixture, 4 µL of 25 mM $MgCl_2$, 5 µL of 10X buffer, 1.5 µL of each of 50 pmol/µL primers, 4 µL of total cDNA, 4 µL of Taq DNA polymerase, 6 µL of nuclease-free water. The amplification started at 90°C for 2 min, followed by 30 cycles of 58°C for 30 seconds, 72°C for 2 min, and final elongation of 72°C for 10 min, at 95°C for 5 min, and at 4°C for 5 min. The *Ce DCXR* cDNA of the PCR product was purified using Qiagen kit columns before cloning.

Cloning Ce DCXR cDNA into TA cloning vectors (Invitrogen 2004)

1) A 10 µL ligation mixture (1 µL of 10X ligation buffer, 2 µL of 25 ng/µL TA cloning vector, 4 µL of 100 ng/µL *Ce DCXR* cDNA (PCR product), 2 µL of sterile water, and 1µL of T4 DNA ligase) was incubated at 14°C overnight; 2) 2 µL of the mixture was mixed with a 50 µL vial of DH5α competent cells, and incubated on ice for 30 min; 3) The vial was heat-shocked in 42°C water for 30 seconds, and immediately transferred to ice; 4) The vial was added 200 µL of SOC media (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose) and incubated at 37°C in a gently shaking incubator for 1 hour; 5) The whole vial was spread on Luria-Bertani (LB) agar plate containing 100 µg/mL ampicillin and 100 µL of 40 mg/mL X-Gal, and incubated overnight at 37°C; 6) Each of 10 white colonies was picked into a 10 ml Eppendorf tube containing 3 mL LB broth added 100 µg/mL ampicillin and incubated at 37°C in a shaking incubator overnight. The recombinant vector of each of three transformed cell lines was isolated by a miniprep protocol using Quaigen Kit columns and the DNA sequence of *Ce DCXR* cDNA in recombinant vector TA (Ce DCXR::TA) was confirmed by the Sanger nucleotide sequencing method using the T7 Promoter Primer.

Ligation of the Ce DCXR sequence in pET28a (+) (Novagen 1999)

A generated cDNA sequence, which was as corrected as predicted, was excised from the recombinant vector TA by the restriction enzymes *Nde*I and *Xho*I, and ligated between the two relevant restriction enzyme sites of pET28a (+). The expected recombinant Ce DCXR::pET28a (+) vector was transformed into the BL21 competent cells. 50 µL of transformation cells was screened on one LB agar media Petri plate (10 cm) which had been already added with 50 µg/mL kanamycin. One cell line which carried Ce DCXR::pET28a (+) was cultured and examined for the *Ce DCXR* sequence by PCR and sequencing before the production of the recombinant Ce DCXR protein. The recombinant Ce DCXR protein was supposed to extend a six-histidine sequence at the N-terminus. The transformed cell line was then cultured in 250 mL of yeast extract tryptone (YT) broth in a shaking incubator at 150 rpm at 30°C overnight until OD_{600} reached 0.5 to 0.7. All cell culture transitions were handled rapidly, the cell suspension was supplemented with IPTG solution to a final concentration of 0.5 mM and cultured on a shaking incubator at 20°C for 4 hours.

The cell suspensions immediately following liquid culture were treated as follows (Novagen 2006): 1) each 250 ml tube of cell suspension was centrifuged at 10,000 rpm using the FA-6X250 Rotor in a Centrifuge 5910 R centrifuge at 4°C for 10 min; The supernatant in the tube was removed, the cell precipitate was retained and then suspended in 1X phosphate-buffered saline (PBS) buffer, called the 2^{nd} cell suspension; 2) The 2^{nd} cell suspension was sonicated for 5 times with each interval of 2 to 3 min and kept cold on ice until it changed from the milky white to the transparent stage; 3) The

sonicated suspension was centrifuged in a cold centrifuge at 12,000 rpm for 10 min. Soon after, the cytosolic fraction on top was transferred to a new tube with a pipette, and the cellular precipitate on the bottom was discarded; 4) The recombinant Ce DCXR in the following cytoplasm was purified using His•Bind kits (Novagen, Protocol TB054 Rev. F0106).

Preparation of anti-GST::Ce DCXR rabbit antibody and immunofluorescence staining

Rabbits were injected with purified glutathione-S-transferase (GST)::Ce DCXR protein (containing GST and all amino acids of Ce DCXR) to produce the polyclonal anti-GST::Ce DCXR antibody. We used the antibody to recognize GST::Ce DCXR, Ce DCXR, and GST proteins using the Western blot technique (Nacalai tesque; Ver.1.2).

pH assays

The buffer solution for catalytic reactions was 0.1 mM NADPH and 0.1 M KH_2PO_4, adjusted to pH 6.0 or 7.0 using HCl or 5N NaOH solutions. The reaction mixture comprising 990 µL of the buffer solution was added with 10 µL of 0.5 M L-erythrulose, 1,4-dibromo-2,3-butanedione, or 0.5 M L-xylulose and 10 µL of 5 mg/mL GST::Ce DCXR at 25ºC. For this assay, the reaction times were recorded at 4, 8, 12, and 16 seconds.

Enzymatic inhibition assays

The catalytic reaction included 2,3-heptanedione, 10 µL of 0.5 mM inhibitor compound, and 10 µL of 0.4 µg/µL Ce DXCR enzyme and was adjusted to a volume of 1 mL with the buffer solution (see the next sentence). 2,3-heptanedione was diluted from 0.6 mM 2,3-heptanedion in the buffer solution to achieve 9 final concentrations of 0.00; 0.02; 0.04; 0.06; 0.08; 0.10; 0.20; 0.40, or 0.60 mM in 9 reactions. The buffer solution was composed of 0.1 mM NADPH, 0.1 M KH_2PO_4, and pH7.0. The reaction times were recorded at 30, 50, 70, and 90 seconds.

Maximum reaction velocity (V_{max}), Michaelis-Menten coefficient (K_m), maximum reaction velocity in the presence of inhibitors (V_{max}^{app}), and the Michaelis-Menten coefficient in the presence of inhibitors (K_m^{app}) in the Michaelis-Menten formula (Jeremy M. Berg *et al.*, 2003) were calculated by the Lineweaver-Burk formula based on the concentration of NADPH and 2,3-heptanedione in the reaction mixture at the given times of the catalytic reaction (Choi *et al.*, 2017). The catalytic degree (K_{cat}) of Ce DCXR was calculated using the extended Michaelis-Menten equation (Srinivasan 2020). The concentration of NADPH in every reaction was determined by OD_{340} in a UV spectrophotometer (Zhang *et al.*, 2000). In this research, the concentration of NADPH was calculated according to the equation: OD_{340} = 4.634286 × [NADPH] + 0.003619. Slopes and intercepts were assumed by the generalized linear model I (GLM) with the formula "summary(lm(1/V~1/[S]]". Statistical analyses were done by using F-statistics for Regression in the R i386 software (R Core Team 2017).

RESULTS AND DISCUSSION

Generation of recombinant Ce DCXR vector

The recombinant *Ce DCXR::pET28a* (+) protein expression vector, which was designed to produce Ce DCXR, was successfully generated. In the vector, a full *Ce DCXR* cDNA coding all 251 amino acids of the Ce DCXR protein was fused with a DNA sequence coding a 6-histidine peptide at the 5' end to become a chimeric *His_6::Ce DCXR* gene. Both the sequence and the reading frame of Ce DCXR in the recombinant vector were precisely ligated and identical to the wild-type *Ce DCXR* cDNA.

Expression and purification of recombinant Ce DCXR

The recombinant vector was transformed in the BL21 competent cells and then conditionally cultured to induce the overexpression of the Ce DCXR enzyme. The wild-type Ce DCXR protein had 28kDa on the

SDS-PAGE gel image (Fig. 1A) and was recognized by the anti-GST::Ce DCXR rabbit antibody (Fig. 1B). These results implicate that the recombinant Ce DCXR protein was successfully produced. The concentrations of the recombinant protein in 5 purified solutions ranged from 2,000 down to 200 ng/µL (Fig. 1A and 1B; the 1st to 5th columns) with a protein purity ($A_{260/280}$) of 0.5 to 0.6, indicating that the quality of the recombinant Ce DCXR enzyme matched with the standard. The purified His_6::DCXR protein solutions were added with glycerol to a final concentration of 15% (v/v) and stored at -20°C for the inhibition assays. However, the Ce DCXR solutions would be contaminated with a little truncated Ce DCXR molecules, which appeared to be smear bands higher than the major band of Ce DCXR in the purified products and also detected by the same anti-GST::Ce DCXR rabbit antibody (Fig. 1B).

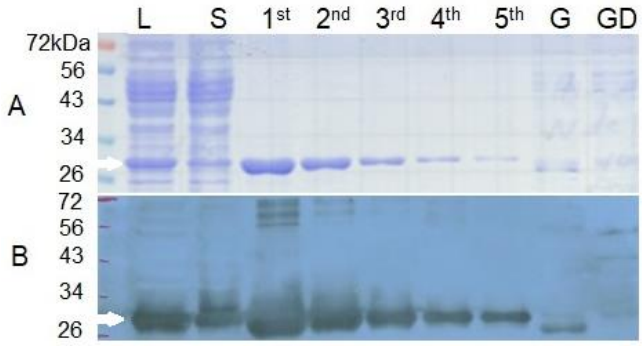

Figure 1. His_6::Ce DCXR expression. (A) Ce DCXR (white array) is separated through SDS-PAGE. L-cell lysate; S - supernatant; 1st to 5th-the 1st to 5th purified His_6::Ce DCXR fractions; G–glutathione S-transferase (GST); GD-GST::Ce DCXR. (B) Western blot. Ce DCXR (white array) is detected by the immunization of the anti-Ce DCXR rabbit antibody.

The pH of catalytic reaction buffer

Because the pH of the catalytic reaction buffer solution regularly affects the enzyme activity, it is necessary to determine the pH scale which could facilitate the catalytic reaction of Ce DCXR. We examined pH 6.0 and 7.0 which are comparable to the intracellular environment pH. We found that pH 6.0 did not reduce OD_{340} of NADPH in the presence of neither substrate L-(+) erythrulose nor 1,4-dibromo-2,3-butanedione, suggesting that the catalytic reactions were not initiated to convert NADPH to NADP+ (Table 1). However, we found a difference at pH 7.0, the OD_{340} of NADPH decreased, suggesting that the catalytic reactions took place in the direction of NADPH oxidation (Table 1). In additional experiments with L-xylulose, the OD_{340} value was also reduced (Table 1). Therefore, we concluded that Ce DXCR performed catalytic reactions at pH 7.0 but not pH 6.0. This was consistent with previous studies which reported that the in situ expression of Ce DCXR was in the cytoplasm where pH was normally neutral (Putnam, 2012).

Inhibition of catalytic reaction

Several derivatives of pyruvic acid, hexanoic acid, and butyric acid were reported to inhibit dehydrogenases. Thus, they and their other derivatives should inhibit Ce DCXR. In this study, we wanted to investigate whether these chemicals or their derivatives may inhibit Ce DCXR, we tested phosphoenolpyruvic, hexanoic acid, and methyl-DL-α-aminobutyrate hydrochloride for inhibiting the catalytic function of Ce DCXR. Therefore, for each chemical, we prepared a reaction mixture consisting of four components (see Methods): Ce DCXR as the enzyme to catalyze the reaction, 2,3-heptanedione as the oxidizing substrate, NADPH as the reducing agent, and a chemical as the tested inhibitor candidate.

Table 1. Chemical kinetics of Ce DCXR at different pH conditions.

		pH 6.0		pH 7.0		
Kinetics	**Substrates**	**L-(+) erythrulose**	**1,4-dibromo-2,3-butanedione**	**L- (+) erythrulose**	**1,4-dibromo-2,3-butanedione**	**L-xylulose**
K_m		N.A	N.A	12.29	12.24	0.095
K_{cat}[a]		N.A	N.A	1.813e-04	4.247-04	2.498-05
K_{cat}[a]/K_m		N.A	N.A	1.47e-05	3.47e-05	2.63e-04

[a]K_{cat} of the reactions were determined at 4 min. N.A-not applicable

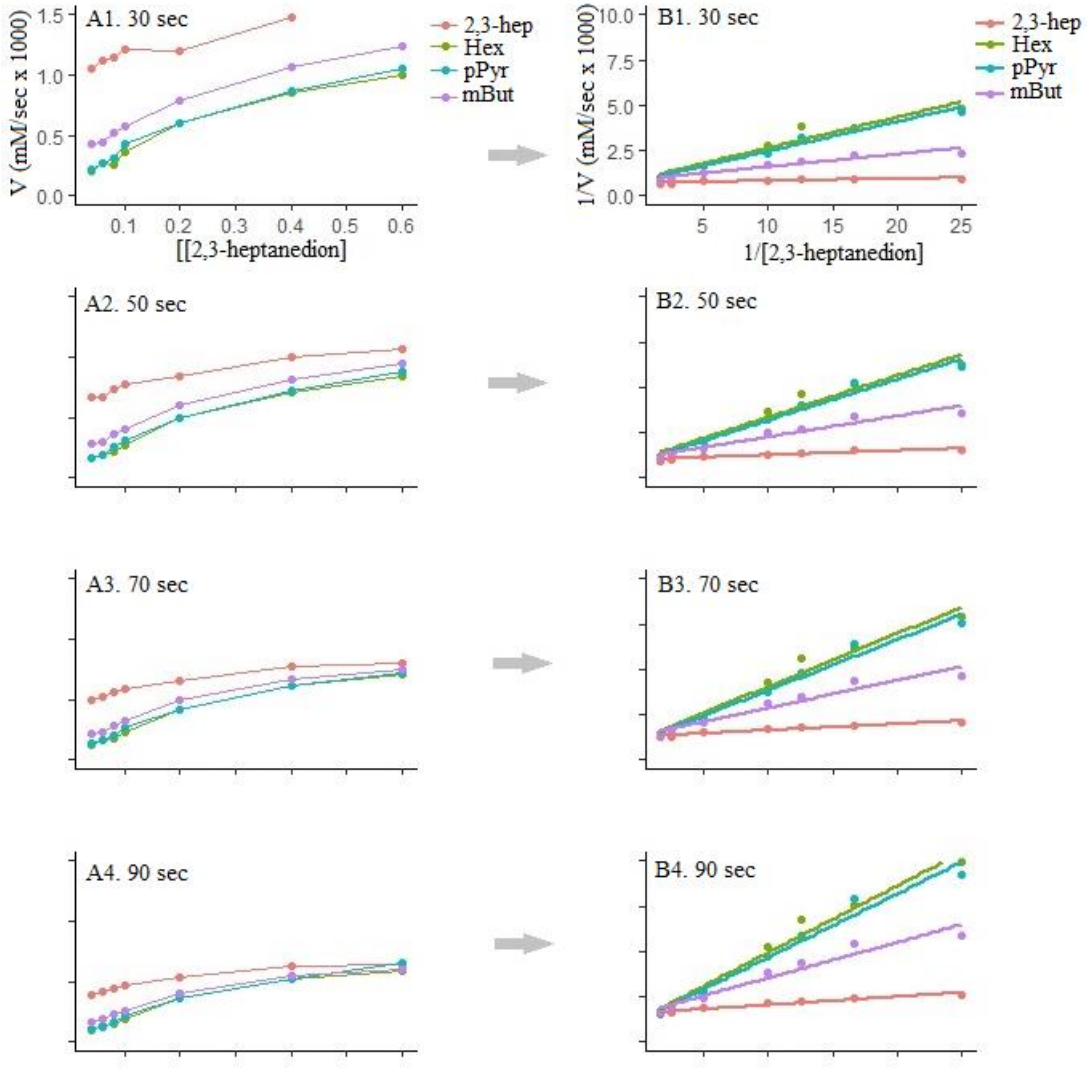

Figure 2. Kinetics of Ce DCXR. A1, A2, A3, and A4 are Michaelis-Menten kinetic graphs at different reaction times (30, 50, 70, and 90 seconds). V (mM/s×1000)) is the substrate conversion rate (mM) for 10^{-3}s. 2,3-hep is the 2,3-heptanedione (red). Hex is hexanoic acid (green). pPyr is phosphoenolpyruvic acid (blue). mBut is methyl-DL-α-aminobutyrate hydrochloride (purple). B1, B2, B3, and B4 are Lineweaver-Burk plots for A1, A2, A3, and A4, respectively. All A subfigures or all B subfigures have the same Y- and X-axis. Arrow-subfigure A relative to subfigure B at each of the reaction times. sec-second.

Table 2. Kinetics of Ce DCXR.

Reaction time (seconds)	Chemical	V_{max} (V_{max}^{app})	K_m (K_m^{app})	R^{2a}	K_{cat}/K_m (K_{cat}/K_m^{app})	(K_{cat}/K_m^{app})/(K_{cat}/K_m) ×100%
30	2,3-hep[b]	1.44	0.20	0.74	585.08	
	Hex[c]	1.06	0.18	0.92	40.04	11.61
	pPyr[d]	1.21	1.00	0.97	41.63	14.06
	mBut[e]	1.15	0.08	0.88	96.26	6.08
50	2,3-hep[b]	1.00	0.02	0.82	278.43	
	Hex[c]	0.96	0.22	0.95	29.60	9.40
	pPyr[d]	1.07	0.24	0.96	30.21	9.22
	mBut[e]	0.91	0.10	0.90	59.04	4.72
70	2,3-hep[b]	0.78	0.03	0.90	195.72	
	Hex[c]	0.88	0.26	0.96	23.23	8.42
	pPyr[d]	0.94	0.12	0.97	24.34	8.04
	mBut[e]	0.75	0.116	0.91	44.40	4.41
90	2,3-hep[b]	0.64	0.03	0.91	149.03	
	Hex[c]	0.79	0.29	0.97	18.59	8.10
	pPyr[d]	0.86	0.30	0.97	19.36	7.70
	mBut[e]	0.64	0.13	0.93	34.58	4.31

[a] R^2 was resulted from GLM to indicate the stage of linear regression correlation. [b] 2,3-hep is the 2,3-heptanedione. [c] Hex is hexanoic acid. [d] pPyr is phosphoenolpyruvic acid. [e] mBut is methyl-DL-α-aminobutyrate hydrochloride.

All reaction kinetics showed the typical patterns of the Michaelis-Menten equation (Fig. 2A1-A4), and the Lineweaver-Burk plot (Fig. 2B1-B4) at different reaction times. They had all V_{max}, V_{max}^{app}, K_m, and K_{max}^{app} greater than zero (F-statistics for Regression, all $P < 0.05$; Table 2). Together, these results indicated that the reaction kinetics are exactly enzymatic types.

In the reactions without potential inhibitor candidates, the K_{cat}/K_m ratios for 2,3-heptanedione were higher and reduced at later reaction times (Table 2). In the presence of every tested inhibitory candidate chemical, the K_{cat}/K_m ratios were extremely lower and much lower at later reaction times (Table 2). In the reactions with hexanoic acid, the K_{cat}/K_m ratio for 2,3-heptanedione was reduced from 8.1 to 11.61 times, suggesting that hexanoic acid was an inhibitor. In the reactions with phosphoenolpyruvic acid, the K_{cat}/K_m was decreased from 7.7 to 14.06 times, indicating that phosphoenolpyruvic acid was an inhibitor. In the reactions with methyl-DL-α-aminobutyrate hydrochloride, it was reduced 4.31 to 6.08 times, suggesting that phosphoenolpyruvic acid was another inhibitor. Taking together, we suggest that three chemicals were inhibitors of Ce DCXR. However, the abilities of inhibition were different among the three inhibitors. Phosphoenolpyruvic acid was the strongest, reducing the enzyme activity (K_{cat}/K_m) 14.06 times, and methyl-DL-α-aminobutyrate hydrochloride was the weakest, reducing 4.31 times.

In this study, we found three new inhibitors for the catalytic activity of Ce DCXR which are hexanoic acid, phosphoenolpyruvic acid, and methyl-DL-α-aminobutyrate hydrochloride. Our findings are consistent with previous reports on pyruvic acid, n-butyric acid, and hexanoic acid

with an inhibitory potency to the DCXR homologs in humans and the suborder Myomorpha (Nakagawa *et al.,* 2002; Ishikura *et al.,* 2003; Sudo *et al.,* 2005). Therefore, we suggest that each of the inhibitors and their derivatives generally inhibit DCXR activity by the same mechanism. Hexanoic acid derivatives served as weapons of plants against predators by inhibiting the predators' enzymes (Wood *et al.,* 1977; Ku *et al.,* 1983; Lopez-Galiano *et al.,* 2017). Thus, if *C. elegans* and many other nematodes could invade plants and they are toxified by the hexanoic acid defense of the plants. Derivatives of butyric acid inhibited DCXRs and other enzymes by binding to the active site of the enzyme. Therefore, DCXRs were not able to catalyze the reaction (O'Connor, Halvorson 1961; Smoot, Pierson 1981; Yasuda, Tochikubo 1985; Mukherjee, Kakkar 1999; Ishikura *et al.,* 2003; Vincenzo Carbone 2004; El-Kabbani *et al.,* 2005; Komata *et al.,* 2005; Dokmanovic *et al.,* 2007; Dokmanovic *et al.,* 2007). Derivatives of pyruvic acid bound to the substrate-binding site of DCXRs and other enzymes. They competed with the substrates and suppressed the catalysis of DCXRs (Das 1937; Montgomery *et al.,* 1956; Nakagawa *et al.,* 2002).

Avoidance of the exposure to phosphoenolpyruvic acid, hexanoic acid, and methyl-DL-α-aminobutyrate hydrochloride and their derivatives should be concerned. If they appear in pharmaceutical products, foods, and drinks, through which they can come into cells, they could inhibit the activity of DCXRs, triggering damage for the host organisms.

CONCLUSION

Our research uncovered: 1) The entire process to successfully produce Ce DCXR; 2) pH 7.0 was appropriate for the catalytic function of the enzyme; 3) Hexanoic acid, phosphoenolpyruvic acid, and methyl-DL-α-aminobutyrate hydrochloride inhibited the catalytic function of the enzyme.

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