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D-Erythrulose Reductase Can Also Reduce Diacetyl: Further Purification and Characterization of D-Erythrulose Reductase from Chicken Liver

Miki Maeda,*,†,[1] Saburo Hosomi,* Tadashi Mizoguchi,* and Tsutomu Nishihara*

**Faculty of Pharmaceutical Sciences, Osaka University, Yamada-oka, Suita, Osaka 565-0871; and †National Institute of Agrobiological Resources, Kannondai 2-1-2, Tsukuba, Ibaraki 305-8602*

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We have discovered new characteristics of D-erythrulose reductase, namely, that it can catalyze reduction of not only D-erythrulose but also such diketones as diacetyl. These substrates have a common structure with two neighboring carbonyls possibly in s-*cis* plane structure, showing that the enzyme may rigorously distinguish between substrates and other compounds. D-Erythrulose reductase was predominantly located in the kidney and the liver of the chicken. The obtained results suggest that D-erythrulose reductase plays an important role in metabolizing α-dicarbonyls in animal organs, because these diketones widely occur in natural foods.

Key words: diacetyl, α-dicarbonyl, diketone, D-erythrulose reductase, tetrose.

Tetriols such as erythritol and threitol are observed in human urine (*1*) and bovine fetal tissues (*2*). In research to establish the metabolic pathway of tetrose and tetriols, D-erythrulose reductase [EC 1.1.1.162] has been purified and characterized from beef and chicken liver by Uehara *et al.* (*3-6*). D-Erythrulose reductase catalyzes the reduction of D-erythrulose to D-threitol in the presence of NAD(P)H. This enzyme can utilize both NADH and NADPH as coenzyme, but NADPH has a much lower K_m value and is preferentially utilized (*4*).

We previously found that, among sugars, D-erythrulose is the only good substrate for D-erythrulose reductase (*4*). We therefore examined the properties of the enzyme in more detail to elucidate the mechanism of rigorous selection of this substrate. In the process, we found that the enzyme could also catalyze diacetyl.

To search for further new substrates, we screened compounds that are structurally related with D-erythrulose and diacetyl. Acetoin, which is structurally similar to D-erythrulose, evinced only low activity. We also examined many carbonyl compounds, classified into five groups: monocarbonyls, α-hydroxycarbonyls, uncharged α-dicarbonyls, charged α-dicarbonyls, and non-vicinal dicarbonyls.

Because diacetyl is used as flavoring in various foods, we investigated the tissue distribution of the enzyme. It was found to be widely distributed in many tissues, and was present kidney and liver in larger quantities. This paper describes the further purification and characterization of the enzyme from chicken liver and suggests a new role of D-erythrulose reductase.

[1] To whom correspondence should be addressed at: National Institute of Agrobiological Resources, Kannondai 2-1-2, Tsukuba, Ibaraki 305. Tel: +81-298-38-7463, Fax: +81-298-38-7417

MATERIALS AND METHODS

Materials—D-Erythrulose was prepared as previously described (*3, 4*). 2′5′-ADP Sepharose 4B was obtained from Pharmacia Fine Chemicals, Sweden. Acetone, diacetyl, acetoin, 2,3-butanediol, isatin, 2,3-pentanedione, pyruvate, and 1,2-cyclohexanedione were purchased from Nakarai Tesque. Methyl pyruvate, ethyl pyruvate, and camphorquinones were from Sigma Chemical. Other chemicals were the highest grade commercially available. Diacetyl was distilled in order to remove other carbonyl compounds and its dimer form. Acetoin was washed with diethyl ether several times to remove the trace of diacetyl.

Purification of the Enzyme—D-Erythrulose reductase was purified from chicken liver by the procedure of Uehara *et al.* (*4*) with two modofications. Our procedure employed five steps: extraction, acetone fractionation, DEAE cellulose column chromatography, hydroxyapatite column chromatography, and 2′5′-ADP Sepharose column chromatography. The first modification is the change of acetone concentration for fractionation from 50-66% to 75%. The second is application of affinity chromatography. The enzyme fraction from the hydroxyapatite column chromatography was applied to a 2′5′-ADP Sepharose 4B column (1.5×4.5 cm) equilibrated with the standard buffer, namely, 2.5 mM sodium phosphate buffer, pH 7.0. The enzyme was eluted with a linear concentration gradient formed from 100 ml of the standard buffer and an equal volume of 0.5 M KCl in the standard buffer. The flow rate was 25 ml/h and 3-ml fractions were collected. The enzyme activity peak was detected at about 150 mM KCl and was separated from a major peak of protein.

Assay of D-Erythrulose Reductase Activity—The enzyme activity was assayed by measuring the decrease in the absorbance at 340 nm due to the oxidation of NADPH. The assay was performed at 25°C in 0.1 M sodium phosphate

buffer (pH 6.3). The reaction mixture contained the enzyme preparation, 2.0 mmol of D-erythrulose and 50 nmol of NADPH in a total volume of 1 ml. One unit of the enzyme activity was defined as the amount of the enzyme that oxidizes 1 μmol of NADPH per minute.

Assay of Reversibility of D-Erythrulose Reductase—Reversibility was measured according to the method of Uehara *et al.* (*4*). The assay mixture contained 300 μmol of substrate, 2 μmol of $NADP^+$, 100 μmol of sodium phosphate buffer, and 1 unit of enzyme in a total volume of 1 ml. The assay was performed at pH 7.5 and 9.0. The reaction was detected by measurement of the increase in the absorbance at 340 nm.

SDS Polyacrylamide Gel Electrophoresis—SDS polyacrylamide gel electrophoresis was carried out on 12.5% polyacrylamide slab gel containing sodium dodecyl sulfate (SDS) and 10 mM 2-mercaptoethanol at pH 8.8 according to the method of Laemmli (*8*). Gels were stained with Coomassie Brilliant Blue R-250 and destained in a mixture of methanol, acetic acid and water (25:8:65).

Identification of Reaction Products—The reaction mixture contained 10 μmol of diacetyl, 30 μmol of NADPH, 0.2 unit of enzyme, and 100 μmol of sodium phosphate buffer, pH 6.0, in a total volume of 1 ml. The mixture was incubated at 37°C, and 150-μl samples were taken after 0, 15, 30, 60, 120, and 240 min. The samples were centrifuged to remove enzyme and desiccated with a large amount of silica gel for 3 days at 20°C. The dried materials were dissolved in 10 μl of methanol and subjected to GS/MS analysis using HP 5890 SERIESII Gas Chromatography-HP 5972 SERIES Mass Selective Detector.

Preparation of Cytosol Fraction from Chicken Organs—Fresh chicken organs were homogenized four times with 2 mM Tris-HCl buffer containing 1 mM EDTA and 150 mM KCl, pH 7.4. The homogenate was centrifuged at 10,000 $\times g$ for 30 min and the supernatant was collected. The supernatant was ultracentrifuged at 104,000$\times g$ for 1 h, and the resulting cytosol fraction was assayed for the enzyme activity.

RESULTS

Purification—Table I summarizes the results of the purification of D-erythrulose reductase from chicken liver. Our modifications of the original purification procedure (*4*) resulted in higher specific activity of the enzyme; about 290 units/mg protein. The enzyme activity was purified 1,700-fold to a single protein band on SDS-PAGE using 12.5% slab acrylamide gel (data not shown). The specific activity was greater by one order although the recovery was 7.4%, which was smaller than in the previous work (*4*).

During the purification procedure, we used a hydroxyapatite column twice. The first time, it was used to remove proteins which had interacted with the column. The second time, it was used to separate other proteins which could not be removed by the previous procedure. Although D-erythrulose reductase was not bound to hydroxyapatite, it was finally purified by the second hydroxyapatite column chromatography.

Uehara *et al.* reported that 2′5′-ADP was the best pyridine nucleotide analogue to protect D-erythrulose reductase from photoinactivation (*6*). Since 2′5′-ADP has been shown to be the best affinity matrix ligand among stable adenine nucleotide analogues, we also used 2′5′-ADP as an affinity matrix ligand and succeeded in separating the enzyme from other proteins.

Substrate Specificity and Kinetic Parameters—We have tested many compounds as substrates for D-erythrulose reductase from chicken liver. They are classified by their structures into five groups: monocarbonyls, α-hydroxycarbonyls, charged and uncharged α-dicarbonyls, and nonvicinal dicarbonyls. The compounds that served as substrates are shown in Table II with their kinetic parameters. Most of the compounds are uncharged α-dicarbonyls; only D-erythrulose and acetoin have different structures. The structures of substrates are given in Fig. 1. It is noteworthy that the enzyme did not catalyze the reduction of 2,4-pentanedione and pyruvate as effectively as that of 2,3-pentanedione and pyruvate esters (data not shown).

As shown in Table II, the K_m value for D-erythrulose, the original substrate, was 1.1×10^{-1} mM. Linear α-dicarbonyl compounds had similar values to each other (between 1×10^{-1} and 5×10^{-1} mM). Pyruvate esters also had almost the same values (about 1 mM). Likewise, 1,2-cyclohexanedione and (1*S*)-(+)-camphorquinone showed similar values (about 7×10^{-1} mM). Phenanthrenequinone had the smallest of all determined K_m values, 2.1×10^{-2} mM, while acetoin had the largest, 1.2×10 mM.

On the other hand, the substrates other than acetoin gave almost the same k_{cat} values as D-erythrulose (from 5×10 to 5×10^2 μmol/min/mg). This shows that the apparent activities are derived from the stability of the enzyme-substrate complex.

Oxidation of Acetoin and 2,3-Butanediol—When the reversibility of the enzyme reaction was assayed at pH 7.5 and 9.0, the oxidation of 2,3-butanediol to acetoin was slightly detected, but the reaction from acetoin to diacetyl was not observed.

Optimum pH Measured with Diacetyl—Effect of pH on D-erythrulose reductase activity was measured with diacetyl. When NADPH was used as the coenzyme, the optimum pH was determined to be between pH 5.8 and 6.0. However, use of NADH gave a wide peak of activity around pH 6.0–6.8.

Identification of Reaction Products—Reaction products were detected by GC/MS analysis. The temperature of GC

TABLE I. **Purification of D-erythrulose reductase from 1 kg of chicken liver.** The protein concentration was measured by the method of Warburg and Christian (*7*), and the enzyme activity was assayed as described in "MATERIALS AND METHODS."

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification (fold)
Acetone fraction	2,050	346	0.169	100	1
DEAE cellulose	207	159	0.768	46.0	5
1st hydroxyapatite	56.9	149	2.62	43.1	16
2′5′-ADP Sepharose	2.89	78.9	27.3	22.8	162
2nd hydroxyapatite	0.11	30.8	288	7.4	1,704

TABLE II. **Kinetic constants for the substrates of D-erythrulose reductase from chicken liver.**

Substrate	K_m (mM)	k_{cat} (μmol/min/mg)
D-Erythrulose	1.1×10^{-1}	3.2×10^{2}
Acetoin	1.3×10	2.1×10^{-2}
Diacetyl	3.9×10^{-1}	1.3×10^{2}
2,3-Pentanedione	2.4×10^{-1}	1.2×10^{2}
3,4-Hexanedione	1.9×10^{-1}	2.0×10^{2}
2,3-Heptanedione	2.6×10^{-1}	1.8×10^{2}
Methyl pyruvate	1.1	7.7×10
Ethyl pyruvate	1.1	1.2×10^{2}
Phenanthrenequinone	2.1×10^{-2}	8.3×10
Isatin	7.2×10^{-1}	2.5×10^{2}
1,2-Cyclohexanedione	6.7×10^{-1}	1.2×10^{2}
(1*S*)-(+)-Camphorquinone	6.8×10^{-1}	6.4×10

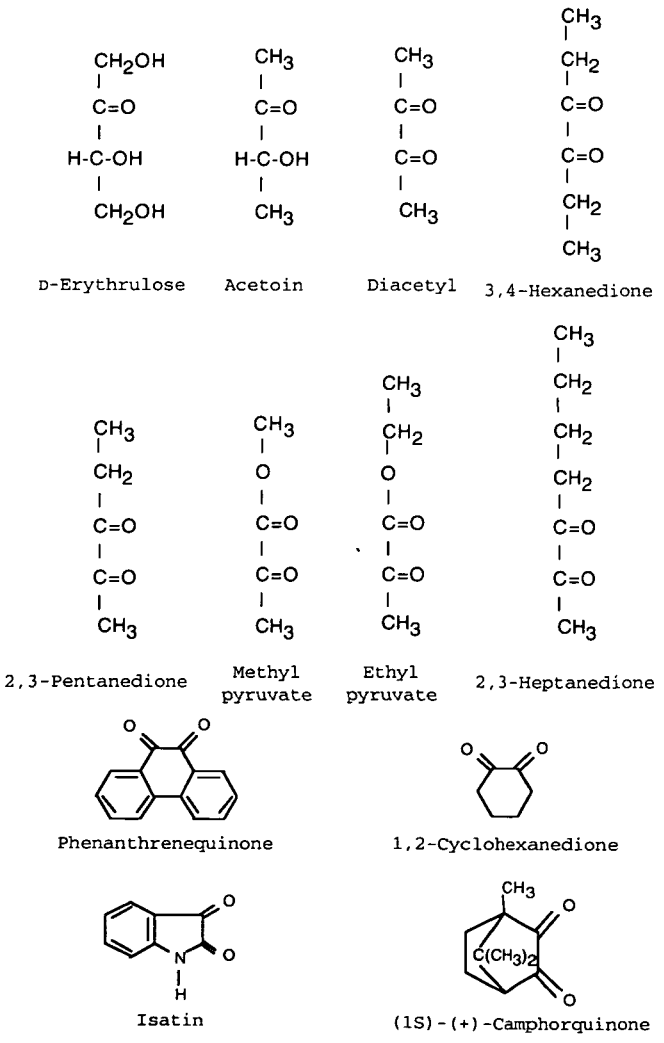

Fig. 1. **New substrates of D-erythrulose reductase.**

was kept at 40°C for 5 min, gradually increased to 50°C over the next 5 min, then rapidly increased to 290°C over the final 8 min. The peak of diacetyl appeared 4 min from the starting point, that of acetoin between 5 and 6 min, and that of 2,3-butanediol between 8 and 10 min.

The peak of acetoin was detected in samples taken at and after 15 min, but it was not observed in the starting sample.

TABLE III. **Tissue distribution of D-erythrulose reductase activity in adult male chicken.** Specific activity means the enzyme activity compared with total cytosol protein.

Organ	Total activity (units)	Specific activity (units/g protein)
Kidney	96.2	33.0
Liver	82.7	17.3
Testis	0.752	16.1
Adrenal	0.053	6.32
Heart	4.30	5.76
Brain	0.464	5.68
Intestine	18.3	4.35
Stomach^{v}	13.2	4.59
Stomach^{p}	2.43	3.48
Pancreas	2.05	2.16
Lung	2.04	1.49
Spleen	0.397	1.23
Lens	0.026	1.06

v, ventriculus; p, proventriculus.

The peak of 2,3-butanediol was not detected in any sample.

Tissue Distribution of D-Erythrulose Reductase—D-Erythrulose reductase activity was measured in several organs of an adult male chicken (Table III). From the specific activity of the purified enzyme (about 290 units/mg), the kidney and the liver were calculated to contain 0.3 mg of D-erythrulose reductase per organ. The kidney gave the highest values of both total and specific activities. The liver had the same total enzyme activity as the kidney, but its specific activity was half that of the kidney. The testis yielded a comparable specific activity to the liver enzyme. The lung, spleen, and lens had the lowest specific activities among the organs tested.

DISCUSSION

Substrate Specificity of D-Erythrulose Reductase—Table II clearly indicates that D-erythrulose reductase can reduce diacetyl as efficiently as D-erythrulose. These two compounds are similar in two ways: (a) both have four carbons and (b) the C-2 has a double bonded oxygen (Fig. 1). Interestingly, acetoin, structurally similar to both D-erythrulose and diacetyl, is a much poorer substrate.

Both D-erythrulose and acetoin have a hydroxyl group at the 3 position, but differ from each other in the functional groups attached to C-1 and C-4. The deletion of the two hydroxyl groups from D-erythrulose, resulting in acetoin, causes a two order increase in K_m value (Table II). This finding indicates that these two hydroxyl groups are necessary for effective binding to the enzyme. However, it is noteworthy that the replacement of these substituents in acetoin with carbonyl (double bonded oxygen) to form diacetyl, also decreased in K_m values (Table II).

Consequently, D-erythrulose reductase catalyzes the reduction of diacetyl substrate to acetoin product, but hardly catalyzes the reduction of acetoin to 2,3-butanediol. The K_m value of 13 mM for acetoin suggests that the enzymatic reduction of this substrate does not occur *in vivo*.

2,3-Pentanedione and 2,4-pentanedione have the same elemental compositions, but the former has two neighboring carbonyls, while the latter does not; and the former is reduced by D-erythrulose reductase while the latter is not. This result indicates that the enzyme can utilize mainly vicinal dicarbonyls.

Methyl pyruvate is structurally similar to 2,3-pentanedione but has oxygen substituted for C-4 (Fig. 1). The K_m value for 2,3-pentanedione is several times smaller than that for methyl pyruvate (Table II), suggesting that a polar atom neighboring the carbonyl group might block the enzyme binding or the reduction.

Chain length of the substrate appeared to have little effect on its reduction. Substrates of the series diacetyl, 2,3-pentanedione, 3,4-hexanedione, and 2,3-heptanedione had similar K_m values, as did methyl pyruvate and ethyl pyruvate.

Isatin is almost as good a substrate as D-erythrulose and diacetyl in terms of its K_m and k_{cat} values, while phenanthrenequinone has a greater affinity for the enzyme than these substrates. Isatin and phenanthrenequinone are planer compounds with two neighboring carbonyl groups that are fixed in s-*cis* form. The high activity with these compounds suggests that this configulation is important.

In 1,2-cyclohexanedione, the dihedral angle between the two carbonyls is more flexible than in phenanthrenequinone and isatin. 1,2-Cyclohexanedione is not as good a substrate as phenanthrenequinone despite the presence of vicinal carbonyls, perhaps because the half-chair form is stable and the two carbonyls are not in s-*cis* conformation. Stereochemical selectivity was observed for other compounds, for example, (1*S*)-(+)-camphorquinone was utilized by the enzyme but (1*R*)-(−)-camphorquinone was not. These results suggest that D-erythrulose reductase has a rigorous stereochemical requirement for interaction with the active site.

In conclusion, D-erythrulose reductase reduces not only D-erythrulose but also some α-dicarbonyl compounds. The s-*cis* plane structure of α-dicarbonyl substrates may be prerequisite for enzymatic reduction.

The Role of D-Erythrulose Reductase—The finding of a large amount of the enzyme in the kidney suggests that it plays an important role there in reducing D-erythrulose to D-threitol.

The specific activity of the enzyme is lower in the liver than in the kidney, but the total amounts of the enzyme are almost equal. This shows that D-erythrulose reductase also plays an important role in the liver. Dicarbonyl compounds such as diacetyl, a good substrate of the enzyme, are present in many everyday foods and beverages, such as alcoholic drinks (*9, 10*), milk (*11*), butter (*12*), buttermilk (*13*), cheese (*14, 15*), and orange juice (*16*). Borders and Riordan reported that about 1 mM diacetyl modified arginine residues in proteins (*17*), which suggests that a high concentration of diacetyl may be toxic for animals. Therefore, the enzyme is considered to detoxify these compounds. Otsuka *et al.* (*18*) proposed a detoxification route from acetaldehyde to 2,3-butanediol β-glucuronide *via* acetoin. D-Erythrulose reductase would produce acetoin by reducing diacetyl, leading to its detoxification and excretion.

The total activity in the testis was lower than in many organs, but the specific activity is in the same as that in the liver. However, we have not detected any substrates in the testis, which suggests that the enzyme may have a different role there.

Another Diacetyl-Reducing Enzyme—The major enzyme which preferentially uses diacetyl as a substrate is designated diacetyl reductase (acetoin:NAD^+ oxidoreductase; EC 1.1.1.5). The enzyme can reduce diacetyl and oxidize acetoin in the presence of NADH (NADPH) and NAD^+ ($NADP^+$), respectively. Diacetyl reductase is widely distributed in organisms from bacteria to animals. However, the enzymes from bacteria (*19-22*) and animals (*22-28*) differ in their substrate specificities. Diacetyl reductase from bacteria can reduce both diacetyl and acetoin, whereas that from animals reduces only α-dicarbonyl compounds. Diacetyl reductase from animals is very similar to D-erythrulose reductase in the following ways: (a) The latter can utilize also α-dicarbonyl compounds in addition to D-erythrulose (Table II). (b) Both enzymes from beef liver are unstable at low temperature (*3, 26*). (c) Both enzymes are homooligomeric proteins with subunit molecular weights of 22,000-28,000 (*3, 4, 22, 25, 26, 28*). These results suggest that diacetyl reductase from animals may be the same enzyme as D-erythrulose reductase.

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