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PURIFICATION AND CHARACTERIZATION OF 2-ALKYNE-1-OL DEHYDROGENASE INDUCED BY 2-BUTYNE-1,4-DIOL IN *FUSARIUM MERISMOIDES* B11

TERUZO MIYOSHI, HIDEO SATO and TOKUYA HARADA*

The Institute of Scientific and Industrial Research, Osaka University, Yamada-kami, Suita-shi, Osaka (Japan)

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SUMMARY

2-Alkyne-1-ol dehydrogenase from the mycelia of *Fusarium merismoides* B11 was purified 320-fold by fractionation with ammonium sulfate followed by chromatographies on *O*-(diethylaminoethyl)-cellulose, calcium phosphate gel and Sephadex G-200.

On ultracentrifugation, the enzyme gave a single, sharp, symmetrical schlieren peak with a sedimentation coefficient ($s_{20,w}$) of 22.3 S. Activity is dependent on both NAD^+ and $NADP^+$, especially the former. The enzyme has an optimal pH of 8.2, and K_m value of $9.1 \cdot 10^{-3}$ M for 2-butyne-1,4-diol.

The general formula for a well-oxidizable substrate is $R–C\equiv C–CH_2OH$, where R may be H, CH_3, CH_2OH and $C\equiv C–CH_2OH$ with the exception of 1,4-butanediol. The activity is sensitive to thiol reagents but not to metal-chelating agents.

The enzyme is only formed when 2-butyne-1,4-diol was used as the sole source of carbon.

INTRODUCTION

Alcohol dehydrogenases (EC 1.1.1.1) are widely distributed in many microorganisms as well as in animal and plant tissues. Many of them have been purified and their properties studied. The abilities of some of these to oxidize acetylenic alcohols have been examined. An alcohol dehydrogenase from a strain of *Pseudomonas* could not oxidize either 2-butyne-1,4-diol or 2-propyne-1-ol (propargyl alcohol) [1]. A dehydrogenase from pea pods was also inactive towards 2-propyne-1-ol [2]. An alcohol dehydrogenase from *Gluconobacter suboxydans* scarcely oxidized 2-butyne-1,4-diol [3]. A crude enzyme from *Botrytis cinera* readily oxidized 2-butyne-1-ol [4] and an inducible allyl alcohol dehydrogenase from *Escherichia coli* oxidized 4-pentyne-1-ol, 2-, 3-, 4- and 5-hexyne-1-ol as well as allyl alcohol (2-pentene-1-ol) [5].

Our previous reports showed that *Fusarium merismoides* B11 can utilize 2-butyne-1,4-diol as the sole carbon source with production of mannitol [6], acetylene

* To whom reprint requests should be sent.

dicarboxylic acid, its esters [7], 2,4,6-triketosuberic acid, 2,4,6,8-tetraketosebacic acid and phthalic acid (Miyoshi, T., Sato, H. and Harada, T., unpublished results).

This paper describes the purification and characterization of a dehydrogenase towards 2-butyne-1,4-diol from this organism.

MATERIAL AND METHODS

Cultures

F. merismoides B11 isolated from soil was used as the source of enzyme. The synthetic medium used for culture contained (per 100 ml) 1 g of 2-butyne-1,4-diol, 0.15 g of $(NH_4)_2HPO_4$, 0.1 g of KH_2PO_4, 0.05 g of $MgSO_4 \cdot 7H_2O$ and 0.01 g each of NaCl, $FeCl_3 \cdot 6H_2O$ and $MnCl_2 \cdot 4H_2O$, and was adjusted to pH 7.0. A conical flask containing 95 ml of the medium was inoculated with 5 ml of a seed culture which had been grown in the same medium. The medium was shaken reciprocally at 30 °C for 2 days. The mycelia were harvested by centrifugation and washed with 0.01 M phosphate buffer (pH 7.0).

Assay

Both NAD^+ and $NADP^+$ were shown to be active hydrogen acceptors using a crude extract of the organisms. Hence alcohol dehydrogenating activity was assayed by measuring the increase in absorbance at 340 nm due to NADH (or NADPH) formation with a Hitachi ultraviolet spectrophotometer, model 124, in conjunction with a Hitachi QPD recorder.

One unit of alcohol dehydrogenase activity was defined as the amount causing formation of NADH (or NADPH) per min and specific activity was defined as units per mg of protein. The protein concentration was determined by the method of Lowry et al. [8] with bovine serum albumin (Sigma Chemical Co.) as standard.

The assay conditions were as follows: To a mixture of 0.1–0.6 ml of enzyme preparation (0.08 unit) and 0.1 ml of 0.1 M aqueous NAD^+ (or $NADP^+$) in 0.1 M buffer of the desired pH (total volume 0.8 ml) was added 0.2 ml of 2 M aqueous substrate in the cell of a spectrophotometer. The reaction was carried out at 30 °C.

Nuclear magnetic resonance spectroscopy

The NMR spectrum of the reaction product was measured with a Hitachi NMR spectrometer, model T-60, in [^{2}H]chloroform using tetramethylsilane as internal standard.

Materials

NAD^+, $NADP^+$ and dithiothreitol were from Sigma Chemical Co. 2-Butyne-1,4-diol, 2-propyne-1-ol (propargyl alcohol), 1-butyne-3-ol, 2- and 3-butyne-1-ol, 2,4-hexadiyne-1,6-diol, *cis*- and *trans*-2-butene-1,4-diol, 1-butene-3-ol and iodoacetate were obtained from Nakarai Chemical Co. *p*-Chloromercuribenzoate was purchased from Wako Pure Chemical Ind., Ltd. Silica gel plastic sheets and silica gel (70–230 mesh, ASTM) for column chromatography were from Eastman Kodak Co. and Merck Co., respectively. All other materials were reagent grade and were obtained from commercial sources.

RESULTS

Purification

All operations were carried out at 5 °C, unless otherwise stated. The phosphate buffer used during the purification steps was prepared by mixing aqueous KH_2PO_4 with aqueous Na_2HPO_4 at the desired concentration, and adjusted to pH 7.5.

Dithiothreitol was added to all solvent systems used for purification (1 mM concentration) as antioxidant, since the dehydrogenase was very sensitive to air (O_2).

Mycelia were harvested by centrifugation from 20 l of cultures from *F. merismoides* B11 (yield, approx. 40 g as dried mycelia). The mycelia were divided into five equal portions. Then the dehydrogenase was purified according to the following steps:

Step 1. One portion of the mycelia was suspended in 240 ml of 0.01 M phosphate buffer and subjected to sonic oscillation for 10 min at 20 kcycles in a 200 W Kaijo Denki Ultrasonic disintegrator. Then the preparation was centrifuged for 60 min at 27 000 × *g*. The crude extract (300 ml) containing approx. 3.6 g of protein, was obtained.

Step 2. The crude extract was brought to 20% saturation of $(NH_4)_2SO_4$. The resulting precipitate was removed by centrifugation. Further solid $(NH_4)_2SO_4$ was added to the supernatant to give 50% saturation. The resulting precipitate was collected by centrifugation and dissolved in 150 ml of 0.01 M phosphate buffer. Then this solution was dialyzed against 4 l of 0.01 M phosphate buffer for 24 h.

Step 3. The dialyzed material was applied to DEAE-cellulose column (5 cm × 15 cm) equilibrated with 0.01 M phosphate buffer. The column was washed with 300 ml of the same buffer and then eluted with 1 l of linear gradient of 0–0.5 M NaCl in 0.01 M phosphate buffer. The fraction of elute containing the enzyme activity (170 ml) was adjusted to 60% saturation of $(NH_4)_2SO_4$, and the precipitate was collected by centrifugation and dissolved in 5 ml of 0.01 M phosphate buffer. The resulting enzyme active solution was dialyzed against 0.01 M phosphate buffer for 24 h.

Step 4. The dialyzed solution was applied to a column (2 cm × 15 cm) of calcium phosphate gel (Hydroxyapatite, Seikagaku Kogyo Co., Ltd), equilibrated with 0.02 M phosphate buffer (pH 7.5). Protein was eluted with 600 ml of linear gradient of 0.02 M to 0.2 M phosphate buffer (pH 7.5).

Step 5. The fraction of eluate containing the enzyme activity (160 ml) was concentrated to 10 ml with a Sartorius-Membranfilter GmbH under vacuum and applied to a column (3 cm × 40 cm) of Sephadex G-200. The column was eluted with 0.01 M phosphate buffer. The active fraction in tubes 26–36 were combined (50 ml). The typical elution pattern is shown in Fig. 1.

The purified enzyme was highly labile, even in the presence of dithiothreitol and only retained activity for 2 days at 5 °C. The purification procedure and specific activity are summarized in Table I.

The overall yield was 26% and the specific activity of the purified enzyme was 751 units per mg. The enzyme was purified 320-fold and about 15 mg of the purified enzyme was obtained from 20 l of culture.

Ultracentrifugal analysis

The sedimentation velocity was measured in a Beckmann analytical ultracentrifuge, model L3-50. The movement of the boundaries was traced on graph paper

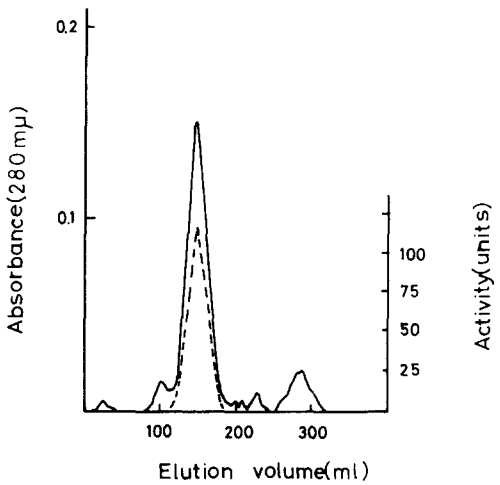

Fig. 1. Gel filtration on Sephadex G-200. The buffer used was 0.01 M phosphate buffer (pH 7.0). The void volume of the column was 50 ml. Absorbance at 280 nm (———); enzyme activity (– – –).

using a photographic enlarger and measured. The sedimentation pattern with the purified enzyme is shown in Fig. 2. The sedimentation coefficient ($s_{20,w}$) was 22.3 S calculated and corrected as described by Schachman [9].

Absorption spectra

The ultraviolet spectrum of the purified enzyme was measured at a protein concentration of 0.5 mg per ml. The absorption maximum in the ultraviolet region was at 277 nm ($E_{1\%}^{1cm} = 13.3$).

Cofactors

The reaction was carried out under the conditions described in Materials and Methods by measuring increase in absorbance at 340 nm due to NADH or NADPH when NAD^+ or $NADP^+$ was added to the reaction mixture as a cofactor. Both NAD^+ and $NADP^+$ were shown to be active hydrogen acceptors for dehydrogenation of 2-butyne-1,4-diol by the purified enzyme, but the former compound was about three times more effective than the latter.

TABLE I

PURIFICATION OF 2-ALKYNE-1-OL DEHYDROGENASE

Activities were measured as described in the text at pH 7.5 in the presence of NAD^+.

Purification step	Volume (ml)	Protein (mg)	Total activity (units)	Spec. act. (units/mg)	Recovery (%)
1. Crude extract	300	3630	8500	2.3	100
2. $(NH_4)_2SO_4$ (0.2–0.5 saturation)	150	1320	7055	5.5	83
3. DEAE-cellulose eluate	170	99	4228	42.7	49
4. Calcium phosphate eluate	160	5	2826	571.0	33
5. Sephadex G-200 eluate	50	3	2253	751.1	26

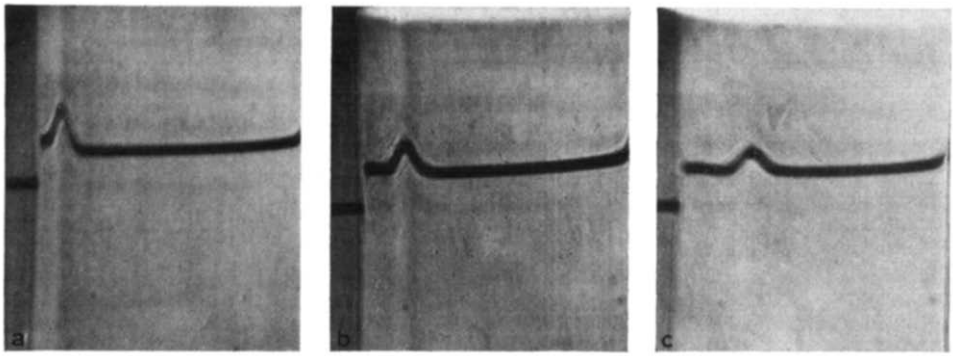

Fig. 2. Sedimentation pattern of 2-alkyne-1-ol dehydrogenase solution containing 5 mg of protein per ml of 0.01 M phosphate buffer (pH 7.0) at 50 000 rev./min at a bar angle of 70°. A single sector cell was used and the temperature was 20 °C. The photographs were taken at (a) 17 min, (b) 25 min and (c) 50 min after attaining 50 000 rpm.

No spectroscopic change was observed at 450 nm when FAD or FMN was added to the reaction mixture instead of NAD^+ or $NADP^+$. Hence, FAD and FMN did not act as cofactors.

Substrate specificity

To characterize the dehydrogenase, the rates of oxidation of various alcohols and glycols with NAD^+ under the conditions specified were measured and the results are shown in Table II. The enzyme was active with all the alcohols tested containing a triple bond, 2-butyne-1-ol, 2-butyne-1,4-diol, 2-propyne-1-ol (propargyl alcohol), 2,4-hexadiyne-1,6-diol, 3-butyne-1-ol and 1-butyne-3-ol, although activity was weak with the latter two compounds. All the good substrates tested above have a triple bond at the α,β-position to the hydroxy group and the primary hydroxy group, while the poor substrates, 3-butyne-1-ol and 1-butyne-3-ol have a triple bond at the β,γ-position and the secondary hydroxy group, respectively. The dehydrogenase showed some activity with all alcohols with a double bond, except for *cis*-2-butene-1,4-diol. Among the saturated alcohols and glycols, 1,4-butanediol, 1,2-propanediol, 1,2,4-butanediol, *n*-propanol, 1,6-hexanediol and 1,2-butanediol acted as substrates.

It is interesting that the activity towards 1,4-butanediol was similar to that towards 2-butyne-1,4-diol. This may be because the mean molecular axis of 1,4-butanediol is linear and so that the spacial orientation of the atoms is probably similar to that in 2-butyne-1,4-diol. This could be true for a relatively high activity towards *trans*-2-butene-1,4-diol as compared to that towards *cis*-2-butene-1,4-diol.

From these results, general formula for all well-oxidizable substrates except 1,4-butanediol is expressed as $R\text{–}C\equiv C\text{–}CH_2OH$, where R may be H, CH_3, CH_2OH, or $C\equiv C\text{–}CH_2OH$. Therefore, this purified enzyme was named 2-alkyne-1-ol dehydrogenase.

The K_m value and V for 2-butyne-1,4-diol were calculated from Lineweaver–Burk plots as $9.1 \cdot 10^{-3}$ M and 4.0 μmole, respectively.

The activity of the crude extracts with the above compounds are also shown in Table III. Its substrate specificity was quite different from that of the purified enzyme

TABLE II

SUBSTRATE SPECIFICITIES OF PURIFIED AND CRUDE ENZYME

Assays were performed in 0.1 M phosphate buffer (pH 7.5), as described in the text, in the presence of NAD^+ as the cofactor. The reaction rate with 2-butyne-1,4-diol was put arbitrarily to 100%.

Substrate	Purified enzyme (relative rate)	Crude enzyme (relative rate)
Methanol	0	0
Ethanol	0	46
n-Propanol	18	93
n-Butanol	0	0
1,2-Ethanediol	0	107
1,2-Propanediol	29	71
1,3-Propanediol	0	8
1,4-Butanediol	94	115
1,3-Butanediol	0	25
1,2-Butanediol	12	36
2,3-Butanediol	0	29
1,2,4-Butanetriol	24	71
1,6-Hexanediol	12	107
1,2,3-Propanetriol (Glycerol)	0	40
2-Propene-1-ol (Allyl alcohol)	18	36
2-Butene-1-ol (mixture of *cis* and *trans*)	8	10
cis-2-Butene-1,4-diol	0	0
trans-2-Butene-1,4-diol	33	115
1-Butene-3-ol	12	55
2-Propyne-1-ol	76	125
3-Butyne-1-ol	6	50
1-Butyne-3-ol	6	321
2-Butyne-1-ol	118	286
2,4-Hexadiyne-1,6-diol	58	42
2-Butyne-1,4-diol	100	100

TABLE III

EFFECTS OF VARIOUS ALCOHOLIC CARBON SOURCES ON DEHYDROGENASE FORMATION

Mycelia grown on various carbon sources for 5 days were oscillated and then centrifuged for 60 min at 27 000 × *g*. The resulting supernatant was assyed for enzyme activity towards 2-butyne-1,4-diol, as described in the text, using NAD^+ as the cofactor at pH 7.5.

Carbon source	Spec. act. (units/mg of protein)
Methanol	0.0
Ethanol	0.1
1,2-Ethanediol	0.4
1,2-Propanediol	0.1
1,3-Propanediol	0.1
1,3-Butanediol	0.3
1,4-Butanediol	0.4
1,6-Hexanediol	0.1
1,2,3-Propanetriol (Glycerol)	0.1
Glucose	0.0
2-Butyne-1,4-diol	6.4
3-Butyne-1-ol	0.3

and, unlike the latter, it showed activity towards 1.2-ethanediol, ethanol, glycerol, 2,3-butanediol, 1,3-butanediol, and 1,3-propanediol. The crude extract showed higher activities towards some substrates relative to that with 2-butyne-1,4-diol than the purified enzyme. Its activity towards 1-butyne-3-ol was especially high. These results indicate that the organism contains another alcohol dehydrogenase when grown on 2-butyne-1,4-diol, although it is uncertain whether an enzyme with high specificity towards alcohols with a triple bond is formed.

Effects of pH and temperature

The optimal pH of the enzyme was shown to be approx. 7.5–8.5 by measuring the dehydrogenase activity at the desired pH using 2-butyne-1,4-diol as the substrate and NAD^+ as the cofactor. The stability of the dehydrogenase in various pH conditions was also examined by measuring activity towards 2-butyne-1,4-diol at pH 7.5 after incubating the enzyme solution of the desired pH at 30 °C for 2.5 h, and the enzyme was shown to be stable between pH 5.8–8.5. The buffers used in these two experiments were as follows: (1) 0.1 M acetate buffer (pH 3.2–5.5) prepared from sodium acetate and acetic acid; (2) 0.1 M phosphate buffer (pH 5.5–7.8) prepared from KH_2PO_4 and Na_2HPO_4; (3) 0.1 M Tris–HCl buffer (pH 7.8–9.1).

The enzyme was shown to be thermally stable below 40 °C by measuring the dehydrogenase activity towards 2-butyne-1,4-diol in the same conditions described above after the enzyme solution was incubated at the desired temperature for 1 h. The activity rapidly decreased above 40 °C and retained 20% of the initial activity at 60 °C.

Effects of additives on 2-alkyne-1-ol dehydrogenase

At concentrations of 2 mM, metal-chelating agents such as EDTA, ethylene diamine and hydroxylamine caused no inhibition, and potassium cyanide and sodium azide slight inhibition. At the same concentrations, the activity was completely lost with thiol reagents such as iodoacetic acid, *p*-chloromercuribenzoate, cupuric chloride and mercuric chloride. These indicate the involvement of an SH group in the action of this enzyme. $NiCl_2$ and $ZnCl_2$ (2 mM) also inhibited the enzymatic activity at the rates of 84% and 55%, respectively.

Isolation and identification of the reaction products from 2-butyne-1,4-diol by the purified 2-alkyne-1-ol dehydrogenase

The reaction followed first-order kinetics suggesting that no side reactions occurred under the reaction conditions.

As a further proof of the nature of the reaction catalyzed by 2-alkyne-1-ol dehydrogenase, the dehydrogenated product from 2-butyne-1,4-diol was isolated and identified as follows. A solution of 50 μmoles of NAD^+, 4 mmoles of 2-butyne-1,4-diol and 180 μg of the dehydrogenase in 10 ml of 0.1 M phosphate buffer (pH 7.5) was incubated for 30 min at 30 °C. Then the reaction mixture was treated with 10 ml of 2,4-dinitrophenylhydrazine (Fredemann and Haugen's reagent [10]). The resulting 2,4-dinitrophenylhydrazone was extracted with 50 ml of ethyl acetate. The solvent was evaporated and the residue was applied to a silica gel column (2 cm × 10 cm) with 200 ml of ethyl acetate as eluting solvent to give a purified 2,4-dinitrophenylhydrazone

as the single reaction product ($R_F = 0.7$ on silica gel thin-layer chromatography, ethyl acetate).

The structure of this hydrazone was deduced by NMR analysis in [^{2}H]chloroform. The protons in this hydrazone were assigned as follows: H_a (9.20 ppm, $J_{a,b} =$ 3Hz), H_b (8.42 ppm, $J_{b,a} = 3$ Hz, $J_{b,c} = 9.6$ Hz), H_c (8.12 ppm, $J_{c,b} = 9.6$ Hz), H_d (10.4 ppm, broad signal), H_e (6.14 ppm), H_f (4.21 ppm) and H_g (2.52 ppm). These results indicate that the dehydrogenated product was 4-hydroxy-2-butynal (HO–CH_2–C≡C–CHO).

Induction of the dehydrogenase

The abilities of the various alcohols listed in Table III to induce 2-alkyne-1-ol dehydrogenase were tested at concentration of 1%. 2-Propene-1-ol, 2-butene-1-ol, *cis*- and *trans*-2-butene-1,4-diol, 1-butene-3-ol, 2-butyne-1-ol, and 2,4-hexadiyne-1,6-diol which have a double bond or triple bond, cannot be utilized as the sole carbon source. Only crude extracts of the organism grown on the medium containing 2-butyne-1,4-diol exhibited high activity. Weak activities were obtained with many compounds, but methanol and glucose did not induce the enzyme. Thus the results showed that 2-butyne-1,4-diol is required for strong induction of 2-alkyne-1-ol dehydrogenase formation.

DISCUSSION

Allyl alcohol (2-propene-1-ol) dehydrogenase from *E. coli* induced by allyl alcohol was reported to show high activity towards some alcohols having a triple bond, as well as towards allyl alcohol [5]. Our organism cannot grow on allyl alcohol or other unsaturated alcohols [6]. The dehydrogenase in this organism is strongly induced by 2-butyne-1,4-diol and has very high specificity towards alcohols with a triple bond at the α,β-position to the primary hydroxyl group. Hence, we named this 2-alkyne-1-ol dehydrogenase.

Another characteristic of this dehydrogenase is its very high molecular weight, which, judging from the sedimentation coefficient (22.3 S), is more than 500 000. On polyacrylamide gel electrophoresis at both pH 9.4 and 4.0, the protein band showed no mobility even on 3.75% gel, especially prepared for protein with a molecular weight of 500 000 [11]. The molecular weight of this dehydrogenase is much greater than those of dehydrogenases from *Pseudomonas* sp. M27 (14 600) [1], liver (84 000) [12] and yeast (150 000) [13], although the dehydrogenase from *Basidiomycetes* [14] is known to have a molecular weight of more than 300 000.

Inhibition experiments indicate that this dehydrogenase has a thiol group. Most alcohol dehydrogenases, including 2-alkyne-1-ol dehydrogenase, probably have a thiol group which is closely related to the dehydrogenase activity with a few exceptions, such as the dehydrogenase [1] from a strain of *Pseudomonas*. A thiol group is thought to be involved in the binding of coenzyme or substrate or both to the alcohol dehydrogenase of liver [15] and yeast [16].

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