Studies on Osmophilic Yeasts.

Part XIV. Partial Purification of Polyol Dehydrogenase from *Pichia* *miso* and the Properties of this Enzyme

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In order to ascertain the existence of polyol dehydrogenase which seemed to take a part in polyalcohol production by *Pichia miso*, the preparation and the partial purification of polyol dehydrogenase from the cells of *Pichia miso* were carried out. Some properties of this enzyme preparation and the identification of the products formed by this enzyme action were also described. This enzyme preparation was found to catalyze the following reactions:

INTRODUCTION

In the previous papers^{1,2)}, the authors reported that *Pichia miso* could completely dissimilate glucose added in the medium at a high concentration such as 30%. The main products were glycerol, erythritol and D-arabitol and the yield of polyalcohols was from 50% to 60% of sugar consumed. Since the mechanism of the polyalcohol production seemed to be worth while for further investigation, the study on polyol dehydrogenase in *Pichia miso* was undertaken.

In the present paper were described the purification procedures of the enzyme, some properties of the enzyme preparation and the identification of reaction products. This enzyme was found to be DPN-specific and catalyzed the following reaction: polyalcohol + DPN⁺ $\rightleftharpoons$ ketose + DPNH + H⁺. The enzyme preparation showed a wide substrate-specificity; no rule was adopted as to the chain

lengths nor to the configurations of hydroxyl groups at carbon 2, 3, 4, and 5 of the substrate. It was also found to be necessary to supplement Fe⁺⁺ for obtaining an active enzyme preparation throughout the course of purification.

The existences of polyol dehydrogenase in mammals³⁻⁵⁾ and microorganisms⁶⁻¹⁷⁾ have been reported. As for yeasts, mannitol dehydrogenase was demonstrated in brewer's yeast⁹⁾ and in *Candida utilis*¹⁸⁾. Edson et

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al.¹⁹⁻²²) extracted various types of polyol dehydrogenases from rat liver, *Acetobacter* and *Pseudomonas*. From the observation on the stereospecificity of the enzymes, they classified them into L-identol dehydrogenase, D-mannitol dehydrogenase and galactitol dehydrogenase according to the Bertrand-Hudson's rule^{23,24}). More recently, the purifications of ribitol dehydrogenase and arabitol dehydrogenase from *Aerobacter aerogenes* were demonstrated by Fromm²⁵) and Lin²⁶) respectively.

EXPERIMENTALS

(1) Preparation of Cell-free Extracts.

Pichia miso was cultivated for 40 hours at 30°C on a reciprocal shaker operating at 120 r.p.m. with a stroke of 75 mm. The composition of the medium was 5% glucose, 0.1% KH_2PO_4 , 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.01% NaCl and 0.4% casamino acid. The cells were collected by centrifugation at 2°C and washed three times with cold 0.05 M Tris buffer, pH 7.2. The washed cells were suspended in a sufficient amount of 0.07 M phosphate buffer, pH 5.6 and starved for 4 hours at 30°C by shaking. The starved cells were harvested and washed three times with cold 0.05 M Tris buffer, pH 7.2. Finally, the cells were spread on a filter paper and dried for 14 hours at room temperature. The dried cells were ground into powder and stored in a desiccator. Approximately 950 g of powdered cells were obtained from about 90 l of broth.

Six g of the powdered cells were suspended in 40 ml of cold 0.05 M Tris buffer, pH 7.2 and exposed to a 10 Kc sonic oscillator for 30 minutes. This sonicated suspension was clarified by centrifuging at $20,000 \times g$ for 15 minutes at 0°C. The clarified extract thus obtained, still contained an appreciable amount of polyols such as 15.7 mg per ml (as glycerol), but only 1.72 mg of protein per ml.

(2) Purification of the Enzyme.

Unless otherwise stated, all the purification processes were carried out below 4°C.

i) Protamine Sulfate Treatment.

To the 650 ml of sonicated extract 4.0 ml of 2% protamine sulfate, pH 7.2, was added dropwise. Mixing was accomplished by gentle magnetic stirring. After standing for 15 minutes, the precipitate was removed by centrifugation at $12,000 \times g$ for 10 minutes.

ii) First Ammonium Sulfate Step.

Fifty two mg of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and 274 ml of ammonium sulfate solution, pH 7.2, saturated at 3°C were added to the protamine treated solution with constant stirring to bring the ammonium sulfate concentration to 30% saturation. After standing for 15 minutes followed by centrifugation at $20,000 \times g$ for 15 minutes, the precipitates were discarded. Additional saturated ammonium sulfate solution was added to the supernatant fluid to bring the saturation to 65%. After standing for 20 minutes, the precipitate was collected by centrifugation ($20,000 \times g$ for 20 min.) and washed twice with 65% ammonium sulfate solution.

iii) Second Ammonium Sulfate Step.

The precipitate thus obtained still contained a small amount of D-arabitol persistently. In order to remove this polyol, ammonium sulfate treatment was repeatedly carried out. The precipitate was dissolved in 0.05 M Tris buffer, pH 7.2, to a final volume of 50 ml. One hundred and twenty mg of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ were added to this solution. After centrifugation the supernatant fraction was mixed with ammonium sulfate solution saturated at 0°C.

The proteins precipitated between 40~55% saturation were collected by centrifugation ($20,000 \times g$, for 15 min), dissolved in 10 ml of 0.05 M Tris buffer, pH 7.2, and then dialyzed against 0.05 M Tris buffer, pH 7.2, containing 10^{-5} M $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ for 4 hours stirring with a magnetic stirrer. The dialyzate containing 19 mg of protein per ml retained its activity during storage for several weeks at -30°C.

(3) Materials.

DPN, DPNH, TPN, diaphorase and D-ribitol were commercial products of Nutritional Biochemical Corporation U.S.A. D-Arabitol and erythritol were produced by the author's procedure¹⁷ from glucose using *Pichia miso* and *Trigonopsis variabilis* under aerobic fermentation.

D-Xylose was prepared by isomerization of D-xylose in boiling pyridine according to the method

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of Schmitt²⁷⁾ and Reichstein²⁸⁾. D-Ribulose was obtained from D-arabinose by a similar procedure.

All other materials were commercial samples.

(4) Determinations.

All spectrophotometric measurements were carried out in a Hitachi Photo-Electric spectrophotometer. DPN, TPN and DPNH were estimated from their absorption spectra at 340 m μ . DPN in the presence of DPNH was determined according to the procedure of Colowick et al.²⁹⁾ using a millimolar extinction coefficient of 5.15 for DPN-cyanide complex at 340 m μ . D-Xylulose in the reaction mixture was determined by the method of Dische and Borenfreund³⁰⁾ after deproteinization with 5% ZnSO₄·7H₂O and 0.3 N Ba(OH)₂. Protein was estimated by the method of Lowry et al.³¹⁾ Ketoses were identified by resorcinol test using the reagents of Kulka³²⁾ and by the orcinol reaction according to Meijbaum³³⁾ except heating for 40 minutes.

(5) Chromatography.

Ketosugars and polyols were separated by ascending-type chromatography using Toyo No. 53 paper. Water-saturated phenol³⁴⁾, containing 0.04% of 8-hydroxyquinoline was used at 20°C and gave satisfactory separations of keto-sugars and polyols. The spots of ketoses were detected with orcinol reagents. Reducing sugars were detected by aniline-hydrogen-phthalate reagent³⁵⁾, which did not react with polyols under the conditions used. *n*-Propanol-ethylacetate-water³⁶⁾ (7:1:2 by volume) was available for the separation of polyols by chromatography. Polyols were detected by spraying with KIO₄ solution and tetrabase-MnSO₄ according to the method of Yoda³⁷⁾.

RESULTS

(1) Oxidation of D-Arabitol and Formation of D-Xylulose.

The partially purified enzyme preparation

in the presence of D-arabitol catalyzed the reduction of DPN to DPNH, but no TPN. This reaction was reversed upon the addition of D-xylulose as shown in Fig. 1.

The product of D-arabitol oxidation was identified as D-xylulose from the following experiments. The reaction mixture was as follows: 7.2 μ moles of DPN, 20 μ moles of D-arabitol, 203 μ moles of Tris, pH 7.2, and 1.0 ml of enzyme solution in a total volume of 3.7 ml. The incubation temperature was 28°±1°.

FIG. 1. Reversible DPN-linked D-Arabitol Dehydrogenase Activity in the Enzyme Preparation of *Pichia miso*.

The reaction mixture (3.7 ml) contained initially 7.2 μ moles of DPN, 203 μ moles of Tris, pH 7.2, 20 μ moles of D-arabitol and 1.0 ml of enzyme preparation.

Incubation temperature: 28°±1° D-xylulose (about 50 μ moles) was added at the point indicated by arrow.

—○— D-Xylulose, —×— TPN
—△— Substrate-free

The product formed from D-arabitol gave an orcinol-ferric color resembling that given by D-xylulose, but different from that of D-ribulose as shown in Fig. 2. The product was identified as well by its positive cysteine-carbazol reaction and resorcinol-ferric test.

The formation of D-xylulose as the product of D-arabitol oxidation was also proved by paper chromatography of an aliquot of reaction mixture. This paper chromatography was carried out by an ascending method with water saturated phenol and the spot was

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FIG. 2. Identification of the Reaction Product Formed Enzymatically from D-Arabitol by Means of Absorption Spectra of the Orcinol-Reaction.

—x— D-Ribulose
—Δ— D-Xylulose
—○— Product formed

detected by spraying with TCA-orcinol and heating at 100°C for 5 minutes. The keto-sugar was identified as D-xylulose by comparing the R_F value and the characteristic gray-blue color with those of authentic D-xylulose. D-Ribulose gave gray-purple color and fluorescent properties when observed under ultraviolet irradiation (short wave 2536 Å).

(2) Stoichiometry of the Reaction.

For the studies on stoichiometry of this enzyme, two experiments were carried out as follows (Table I and II).

Assay 1, the reaction mixture, containing 7.2 μ moles of DPN, 20 μ moles of D-arabitol, 300 μ moles of Tris, pH 7.2, and 0.7 ml of enzyme solution in a total volume of 6.0 ml, was incubated for 45 minutes at 28°±1°.

Assay 2, the reaction mixture contained 3.13 μ moles of DPNH, 3.31 μ moles of D-xylulose, 113 μ moles of Tris (pH 7.2) and 0.7 ml of enzyme solution in a total volume of 2.5 ml; the mixture was incubated for 30 minutes at 29°C. The reaction velocity of D-xylulose reduction (assay 2) was found to be much faster than that of D-arabitol oxidation (assay 1).

TABLE I. STOICHIOMETRY OF D-ARABITOL DEHYDROGENASE ACTIVITY IN THE PREPARATION.

(Assay 1)

	0 min.	45 min.	Δ
	μ moles	μ moles	μ moles
DPN	7.20	7.024	0.176
D-Arabitol	20.00	*(0.137)	*(0.137)
DPNH	0.00	0.181	0.181
D-Xylulose	0.00	0.137	0.137

* estimated from formation of D-Xylulose.

The reaction mixture initially contained 7.2 μ moles of DPN, 20.0 μ moles of D-Arabitol, 300 μ moles of Tris, pH 7.2 and 0.7 ml of enzyme solution in a total volume of 6.0 ml.

After 45 minutes, incubation at 30°C, the reaction was stopped by boiling the mixture at 100°C for 2 minutes. Boiled enzyme was used as control.

Assays were made as follows:

D-Xylulose: cystein-carbazole method.

DPNH: at E 340, pH 7.2 (6.3×10^3)

DPN: the method of Colowick et al.²⁹, with KCN (pH 11.0)

TABLE II. STOICHIOMETRY OF D-ARABITOL DEHYDROGENASE ACTIVITY IN THE ENZYME PREPARATION. (Assay 2)

	0 min.	30 min.	Δ
	μ moles	μ moles	μ moles
DPNH	3.13	1.70	1.43
D-Xylulose	3.31	1.74	1.57
DPN	0.00	1.42	1.42
D-Arabitol	0.00	*(1.57)	*(1.57)

* estimated from disappearance of D-Xylulose.

The reaction mixture initially contained 3.13 μ moles of DPNH, 3.31 μ moles of D-Xylulose, 113 μ moles of Tris, pH 7.2 and 0.7 ml. of enzyme solution in a total volume of 2.5 ml.

As shown in Table II, considerable amounts of D-xylulose and DPNH disappeared in parallel while in Table I, the formation of only small amounts of these compounds was observed. Fig. 3 is a chromatogram showing the formation of D-arabitol from D-xylulose in Assay 2; the paper chromatography was carried out by developing the deproteinized reaction mixture with *n*-propanol-ethylacetate-water³⁶⁾ (7:1:2) and the spot of D-arabitol produced was detected by the method of Yoda³⁷⁾.

(3) pH Dependence.

The influence of pH values on D-arabitol oxidation by the enzyme preparation was observed. The reaction mixtures initially

FIG. 3. Formation D-Arabitol from D-Xylulose.
(The Reaction Mixture in Assay 2).

a: D-Ribulose. b: D-Xylulose. c: Reaction mixture (initial).
d: Reaction mixture (after incubation for 35 min.). e: D-Arabitol.
f: D-Xylitol. g: D-Ribitol.

contained 20μ moles of D-arabitol, 7.02μ moles of DPN, 1.0 ml of enzyme preparation in a total volume of 4.0 ml using several kinds of buffers. The incubation was carried out for 30 minutes at 30°C . Keto-pentose produced was estimated by the cystein-carbazol method. As shown in Fig. 4, the optimal pH of this reaction was 7.0, and the reaction was not affected by the kinds of buffers used.

FIG. 4. D-Arabitol Dehydrogenase Activity of the Enzyme Preparation as a Function of pH.

○: Acetate buffer
△: Phosphate buffer
×: Tris buffer

(4) Substrate Specificity.

Oxidation reaction by this enzyme was ex-

amined on several polyalcohols such as D-mannitol, D-sorbitol, D-xylitol, D-ribitol, erythritol, glycerol etc. beside D-arabitol.

As the result, all the polyols tested were oxidized by the same enzyme preparation to the corresponding keto-sugars. The enzyme preparation oxidized even ethanol.

FIG. 5. Substrate Specificity of the Enzyme Preparation (1).

—△— Ethanol
—×— Glycerol
—○— D-Ribitol
—●— Erythritol
—▲— D-Arabitol
—□— D-Mannitol
— — Substrate-free

FIG. 6. Substrate Specificity of the Enzyme Preparation (2).

—×— D-Sorbitol
—○— D-Dulcitol
—△— D-Xylitol

Figs. 5 and 6 show the specificity of this enzyme towards polyols and ethanol. Ethanol, glycerol and D-ribitol are oxidized rapidly, while the erythritol, D-arabitol and D-mannitol are oxidized slowly. Oxidation velocities of D-xylitol, D-dulcitol and D-sorbitol are in the intermediate of these two groups.

(5) Identification of Reaction Products from Polyols.

In order to determine ketoses formed from various polyols, the following reactions were carried out. The reaction mixtures containing polyols (20μ moles), Tris (188μ moles), DPN (14.4μ moles) and 0.7 ml of enzyme solution in a total volume of 3.7 ml were incubated for 2 hours at $29^\circ \pm 1^\circ$. After deproteinization with 5% ZnSO_4 and 0.3 N $\text{Ba}(\text{OH})_2$, products were identified as follows, and the results were summarized in Table III.

D-Mannitol was oxidized to a product which gave a red resorcinol reaction with two absorption maxima at $410 m\mu$ and $470 m\mu$. Chromatography (phenol-water) gave a single spot of ketose which was identical to that of authentic D-fructose. The product was found to be D-fructose. Similarly, a reducing substance formed from D-sorbitol was determined as D-fructose.

Dulcitol produced a sugar which gave a red resorcinol test. Chromatography of the sugar showed a single spot differing from

fructose or sorbose. The product possibly D-tagatose.

D-Ribitol formed a reducing sugar which gave a green-blue resorcinol reaction with two absorption maxima at $420 m\mu$ and $600 m\mu$. With the use of TCA-orcinol reagent, chromatography gave a fluorescent spot under ultraviolet irradiation. By the color and the R_F value, the product was distinguished from D-xylulose and found to be D-ribulose.

D-Xylitol was oxidized to a keto-sugar which gave a green resorcinol test with two absorption maxima at $430 m\mu$ and $660 m\mu$. In chromatography, the spot gave gray-blue color and R_F value of 0.59 by using TCA-orcinol as spray reagent, the properties being identical to those of D-xylulose.

Erythritol produced yellowish green color in the resorcinol test. Chromatography gave a single spot having R_F value of 0.69~0.70. It might be erythrulose.

Glycerol was oxidized to dihydroxyacetone which gave pink-red color in the resorcinol reaction.

(6) Heat Inactivation of the the Enzyme.

The purified enzyme preparation was stored at -30°C for several weeks without any loss of activity.

At 0° , the enzyme lost half the activity

TABLE III. IDENTIFICATION OF THE REACTION PRODUCTS FORMED ENZYMATICALLY FROM VARIOUS POLYALCOHOLS

Substrate	R_F values of keto-sugar produced	Reduction test	Resorcinol reaction color	Identification of oxidized product
None	—	negative	—	—
Ethanol	—	—	—	Acetaldehyde?
Glycerol	—	positive	ketotriose, pink-red	Dihydroxyacetone
Erythritol	0.68	positive	ketotetrose, yellowgreen	erythrulose?
D-Xylitol	0.56	positive	ketopentose, green	D-xylulose
D-Ribitol	0.61	positive	ketopentose, greenblue	D-ribulose
D-Arabitol	0.56	positive	ketopentose, green	D-xylulose
D-Sorbitol	0.55	positive	ketohexose, red	D-fructose
D-Dulcitol	0.46	positive	ketohexose, red	D-tagatose?
D-Mannitol	0.54	positive	ketohexose, red	D-fructose

after 72 hours (pH 7.2).

After 72 hours at 20°C (pH 7.2), the loss of activity became much greater. The degrees of reduction of enzymatic activities on exposure to 50°C for 8 minutes at pH 7.2, are shown in Table IV.

TABLE IV. HEAT INACTIVATION ON THE ENZYME PREPARATION

Substrate	Increase of absorption at 340 m μ		Residual activity	Activity inactivated
	Heated	Not heated		
Ethanol	.008	.094	8.5%	91.5%
Glycerol	.008	.093	8.6	91.4
Erythritol	.007	.041	17.1	82.9
D-Xylitol	.006	.059	10.2	89.8
D-Ribitol	.007	.082	8.5	91.5
D-Arabitol	.005	.037	13.5	86.5
D-Sorbitol	.006	.082	7.3	92.7
D-Dulcitol	.008	.071	11.3	88.7
D-Mannitol	.004	.027	14.8	85.2

The activity was defined as an increase in optical density at 340 m μ between 4 and 5 minutes of incubation. The reaction mixture initially contained 7.2 μ moles of DPN, 20 μ moles of substrate, 203 μ moles of Tris (pH 7.2) and 0.7 ml of enzyme solution in a total volume of 3.7 ml. It was revealed that this enzyme preparation was very labile to high temperature, and that by heating the enzyme at 50°C for 8 minutes, about 90 per cent of dehydrogenation activities were lost regardless of substrates.

(7) Effects of Metal Ions on Enzyme Activity.

It was found to be necessary for maintaining the enzyme activity to supplement Fe⁺⁺ ions throughout the course of purification. Unless Fe⁺⁺ was present at all steps of purification, the dialyzate showed scarcely any activity even when Fe⁺⁺ was added afterwards as shown in Fig. 7.

FIG. 7. Effect of Metal Ions on the Enzyme Activity.

—○— Supplemented with Fe⁺⁺ during the full course of enzyme purification.
 —×— Not supplemented with Fe⁺⁺ (dialyzed for 4 hours)
 —△— Not supplemented with Fe⁺⁺ (dialyzed for 16 hours)

This fact suggests that the enzyme protein is subjected to the irreversible denaturation in the absence of Fe⁺⁺. From the examination of effects of many other metal ions on the enzyme activity, it was revealed that all activities were lost instantly by addition of Cu⁺⁺ or Zn⁺⁺ ions.

Cu⁺⁺ led to complete inactivation or denaturation of the enzyme, but inhibition by Zn⁺⁺ was partially reversed after one hour of contact with 10⁻³ M of Fe⁺⁺.

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